ENDOSPERM CULTURE OF COCONUT

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ABSTRACT

The growth and differentiation of endosperm tissue of coconut cv. Samoan Dwarf *in vitro* were described. Endosperm tissues produced callus profusely without any enclosing embryo. Explants formed callus three weeks after culture.

Callogenesis occurred in over 95% of all treatments. There was no significant difference in callogenesis between fruit sources, antipodal and micropylar tissues, 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-amino-3,5,6-trichloropicolinic acid (picloram) as well with 6-benzylaminopurine (BAP). Auxin and cytokinin were not necessary to initiate callogenesis.

Growth rate was influenced by fruit source and concentration of 2,4-D and picloram but not by endosperm region of fruit, two types of auxin (2,4-D and picloram) and addition of BAP. Growth rate of tissues increased substantially between 9 to 25 weeks but decreased by 31 weeks of culture. 2,4-D and picloram (10^{-3}M) inhibited growth rate in the beginning. The growth rate of the control was greater than other treatments at 31 weeks after culture.

Tissue (endosperm and callus) browning occurred but did not inhibit tissue growth. In growth, tissue color changed from yellowish white, brown to black from which new yellowish white callus was produced. Callus structure changed from compact to friable after several transfers.

Morphogenesis occurred in endosperm callus of antipodal tissue initially treated with 10^{-6}M picloram after 21 weeks of culture. This "organ" was elongate, opaque and grew slowly. Its shape changed from triangular with several lumps on the surface to cylindrical after 14 months. Morphogenesis also occurred on endosperm callus treated with 207.04×10^{-6}M picloram after 17 months.

Histological study of endosperm callus showed structures which resembled proembryos, embryos with suspensors, promeristemoids and meristemoids. While "organ-like" structure showed a meristematic layer with a dermal layer, cortex-like region and central vascular tissue; there were many small protuberances which resembled embryoids and shoot with tunica and corpus. Callogenesis and morphogenesis (shoot organogenesis) occurred in coconut endosperm *in vitro*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (continued)

Table page

LIST OF FIGURES

Figure page

LIST OD FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS

ABA	abscisic acid
AC	activated charcoal
AdS	adenine sulfate
AZI	7-azaindole
BAP	6-benzylaminopurine
BM	basal medium
CH	casein hydrolysate
CW	coconut water
2,4-D	2,4-dichlorophenoxyacetic acid
DAP	days after pollination
EC	embryogenic cells
GA	gibberellic acid
GLM	General Linear Model
HCHO	paraformaldehyde
HR	historesin
IAA	indole-3-acetic acid
IAA-ala	IAA-L-alanine
IAA-asp	IAA-L-aspartic acid
IBA	indole butyric acid
IKI	potassium iodide-iodine
2iP	6-(γ-γ-dimethylallylamino) purine
IPA	indole-3-propionic acid
KN	kinetin
MS	Murashige & Skoog
ME	malt extract

LIST OF ABBREVIATIONS (continued)

NAA	α-naphthaleneacetic acid
NEC	nonembryogenic cells
NOA	2-naphthoxy acetic acid
PAS	periodic acid Schiff
PCMP	2(p-chlorophenoxy) 2-methylpropionic acid
Picloram	4-amino-3,5,6-trichloropicolinic acid
PPM	part per million
RCBD	randomized complete block design
SAS	Statistical Analysis System
SE	somatic embryogenesis
TIBA	2,3,5-triiodobenzoic acid
YE	yeast extract
W	White
WOC	weeks of culture

CHAPTER I

INTRODUCTION

Coconut (Cocos nucifera L.) belongs to the Arecaceae family and is distributed in tropical areas. There are approximately 11 million ha of coconuts growing in more than 80 countries of the tropics (Thangaraj and Muthuswami, 1990; Persley, 1992). Most belong to small holders who grow them for domestic use (Benbadis, 1992). The various common names given to coconut reflect its usefulness, e.g. tree of life, tree of abundance, tree of heaven (Green, 1991). All parts of the plant are very useful for humankind (Menon and Pandalai, 1957; Reynolds, 1982; Blanton and Blake, 1983b; Thangaraj and Muthuswami, 1990; Green, 1991; Persley, 1992). The plants are also used as an ornamental plant in regions such as Hawaii and Florida.

The plant is traditionally propagated by seed which is recalcitrant and has a short storage life. The plant is long-lived but has a very long juvenile phase. It is generally cross pollinated and very heterozygous (Tammes and Whitehead, 1969; Reynolds, 1982; Blake, 1983; Bhaskaran, 1985; Karunaratne and Periyapperuma, 1989). Therefore vegetative propagation is needed in order to have uniform plants.

Although micropropagation in vitro from various tissues

(embryo, stem, leaf, root, inflorescence, endosperm, and anther) have been attempted, success is mostly limited to immature unselected plants producing very few plants (Brackpool et al., 1986; Pannetier and Buffard-Morel, 1986; Blake, 1991). Micropropagation of other palms, oil and date palms, has been successful (Tisserat, 1979; Corley et al., 1981; Sharma et al., 1984; Rao and Ganapathi, 1993). A reliable method of clonal propagation of coconut would be a great asset for breeding program due to fast multiple of selected plant in relatively short time (Blake, 1991).

Coconut endosperm provides large and uniform explant tissue for experimentation. Micropropagation of endosperm tissue would be advantageous since explants come from mature selected plants. Plants from endosperm may be triploids and may produce fruits without embryos or produce no fruit. If they bear no fruit, it will have a secondary advantage for Hawaii and other areas where it is used for ornamental purposes since large fruits must be removed to avoid the dangers encountered by falling fruits.

The objectives of this study are twofold: first, to establish a protocol for initiation in vitro of callogenesis from endosperm of mature selected plants and second, to induce morphogenesis and ultimately plant regeneration.

CHAPTER II

LITERATURE REVIEW

Some methods of vegetative propagation have been applied in coconut. Airlayering of tall trees by attaching a wooden box filled with sand or coir dust on the wounded stem, results in a rooted tree that can be planted. This reduces tree height and increases production (Davis, 1969). Airlayering of bulbils emerging abnormally within the spathe instead of an inflorescence produces only a limited number of plants. These plants at maturity are sterile because only bulbils instead of inflorescence are produced (Davis, et al., 1985). Splitting the growing point of stem results in more than one shoot (Davis, 1969; Fisher and Tsai, 1979). None of these methods is satisfactory. Thus there is a need to pursue *in vitro* multiplication of coconut.

In vitro studies of coconut started in the early 1950's with embryo culture (Cutter and Wilson, 1954). Researchers in the Philippines regenerated plants from zygotic embryos of "Makapuno" cultured *in vitro* (Balaga and De Guzman, 1971; De Guzman et al.,1971; De Guzman and Manuel, 1977) and in Indonesia from embryos of "Kopyor" which is similar to "Makapuno" (Tahardi, 1987b). "Makapuno" or "Kopyor" has a gelatinous endosperm which is highly valued as a delicacy in drinks or ice cream (Tammes and Whitehead, 1969). Although

the embryo is fully developed, it cannot be grown from seed due to disfunction of the endosperm (Tammes and Whitehead, 1969; De Guzman, 1981).

Plants of other coconuts have also been propagated through embryo culture (De Guzman et al., 1978; D'Souza, 1982; Gupta et al., 1984; Bah, 1986; Bhalla-Sarin et al., 1986, and Sugimura et al., 1988). Tahardi (1987a) reported that only roots grew from sliced embryos of coconut. Plantlets were regenerated through callogenesis and embryogenesis from 6 - 7 month-old coconut embryos in vitro (Karunaratne and Periyapperuma, 1989).

Others reports of in vitro studies of coconut include cultures of cotyledon (Jagadeesan and Padmanabhan, 1982), stem apices (Blake and Eeuwens, 1982; Branton and Blake, 1983a; Gupta et al., 1984), leaves (Pannetier and Buffard-Morel, 1982a; Raju et al., 1984; Verdeil et al., 1989; Buffard-Morel et al., 1992), inflorescences (Blake and Eeuwens, 1980; Branton and Blake, 1983a; 1986; Gupta et al., 1984; Sugimura et al., 1988; Sugimura and Salvana, 1989; Verdeil et al., 1989), endosperms (Fisher and Tsai, 1978; Bhalla-Sarin and Bagga, 1983; Kumar et al., 1985), roots (Bhalla-Sarin and Bagga, 1983; Schwabe, 1983) and anthers (Thanh-Tuyen and De Guzman, 1983; Monfort, 1985). Explants from these tissues, especially leaves and inflorescences produced embryoids and plantlets. Table 1 is a summary of coconut cultures reported from various tissues.

Table 1. Coconut cultures reported from various tissues.

Explant source	Growth regulator	Response	Reference
Embryo	57.08-114.16x10^{-6}M IAA	shoot and root	De Guzman et al., 1971
	0.54x10^{-6}M NAA	plantlets	De Guzman et al., 1978
	26.85-40.28x10^{-6}M NAA	callus	D'Souza, 1982
	without	bud-like structure	
	5.37-10.74x10^{-6}M NAA	root and pneumatophores	
	12-20x10^{-6}M 2,4-D	callus	Karunaratne and Periyapperuma, 1982
	2-8x10^{-6}M 2,4-D + 10^{-5}M BAP and KN each	embryoids and plantlets	
	8.12x10^{-6}M IAA-ala or 6.89x10^{-6}M IAA-asp	callus	Bhalla-Sarin and Bagga, 1983
	6.89x10^{-6}M IAA-asp + 9.29x10^{-6}M KN or 2.69x10^{-6}M NAA + 8.87x10^{-6}M BAP	shoot and root	
	2.26-22.6x10^{-6}M 2,4-D + 0.05-5.37x10^{-6}M NAA + 0.44-4.44x10^{-6}M BAP	shoot and root	Gupta et al., 1984

Table 1. Coconut cultures reported from various tissues (continued).

Explant source	Growth regulator	Response	Reference
Embryo	without	shoot and root	Karunaratne et al., 1985
	without	shoot and root	Bah, 1986
	10.74×10^{-6}M IAA-asp or 8.12×10^{-6}M IAA-ala	callus	Bhalla-Sarin et al., 1986
	6.89×10^{-6}M IAA-asp + 9.29×10^{-6}M KN or 10.74×10^{-6}M NAA	plantlet	
	without	shoot and radicle	Tahardi, 1987b
	57.08×10^{-6}M IAA	shoot and root	
	452.50×10^{-6}M 2,4-D +BAP + 2iP	callus and root-like structure	Sugimura et al., 1988
	$100\text{-}300 \times 10^{-6}$M NAA	shoot and root	Ashburner et al., 1993
Cotyledon	4.44×10^{-6}M BAP + 2.69×10^{-6}M NAA	callus	Jagadeesan and Padmanabhan, 1982
	4.44×10^{-6}M BAP + 5.37×10^{-6}M NAA	rootlets and pneumatophores	

Table 1. Coconut cultures reported from various tissues (continued).

Explant source	Growth regulator	Response	Reference
Stem apice	100^{-5}-10^{-7}M 2,4-D	plantlets	Blake and Eeuwens, 1982
	$2.5x10^{-4}$M 2,4-D + AC or $2.5x10^{-6}$M 2,4-D	roots	
	10^{-4}M 2,4-D + $5x10^{-6}$M BAP + $5x10^{-6}$M 2iP	callus	Branton and Blake, 1983a
	10^{-8}M 2,4-D + $5x10^{-6}$M BAP + $5x10^{-6}$M 2iP	embryoids	
	$4.52x10^{-4}$M 2,4-D	callus	Gupta et al., 1984
	$0.27x10^{-6}$M NAA + $2.22x10^{-6}$M BAP + $0.93x10^{-6}$M KN	embryo-like structure	
Leaf	$2.26x10^{-4}$M 2,4-D	embryo-like structure	Gupta et al., 1984
	KN, 2iP, BAP + NAA, IAA, IPA	callus and embryoids	Raju et al., 1984
	withdrawal NH_4, HCHO, CH, reduced auxin to 75% and increased CK to 125%	plantlets	
	auxin	callus	Verdeil et al., 1989
	reduce gradually auxin	embryoids and plantlets	

Table 1. Coconut cultures reported from various tissues (continued).

Explant source	Growth regulator	Response	Reference
Leaf	1.35-2.70x10^{-4}M 2,4-D	embryo-like and 1 shoot and root	Buffard-Morel et al., 1992
	0.22-0.45x10^{-4}M 2,4-D		
Inflores-cence	2.5x10^{-7}M NAA + 5x10^{-6}M BAP to 2.5x10^{-6}-2.5x10^{-5}M NAA + 5x10^{-8}M BAP	shootlet and root	Blake and Eeuwens, 1980
	10^{-4}M 2,4-D + 5x10^{-6}M 2iP + 5x10^{-6}M BAP + 2.7x10^{-6}M AdS	callus	Branton and Blake 1983a; 1986
	10^{-8}M 2,4-D + 5x10^{-6}M 2iP + 5x10^{-6}M BAP + 2.7x10^{-6}M AdS	embryoids and 2 plantlets	
	4.52x10^{-4}M 2,4-D	callus	Gupta et al., 1984
	2.3x10^{-5}M 2,4-D	root and shoot-like structure	
	90.50-109x10^{-6}M 2,4-D + BAP + 2iP	callus	Sugimura et al., 1988; Sugimura and Salvana, 1989
	4.52-22.62x10^{-6}M 2,4-D + BAP + 2iP	shoot-like structure or root	
	auxin	callus	Verdeil et al., 1989
	reduce gradually auxin	embryoids	

Table 1. Coconut cultures reported from various tissues (continued).

Explant source	Growth regulator	Response	Reference
Endosperm	10.74×10^{-6}M NAA + 9.29×10^{-6}M KN	no	Bhalla-Sarin and Bagga, 1983
	5.71×10^{-6}M IAA + 4.65×10^{-6}M KN	callus	Fisher and Tsai 1978
	$10.7\text{-}26.8 \times 10^{-6}$M NAA	continue growth	
	2.3×10^{-4}M 2,4-D + 9.3×10^{-6}M KN	callus	Kumar et al., 1985
	4.5×10^{-6}M 2,4-D + 2.3×10^{-6}M KN	continue growth	
Root	9.05×10^{-6}M 2,4-D or 9.80×10^{-6}M IBA	roots or callus	Schwabe, 1983
	6.89×10^{-6}M IAA-asp + 8.87×10^{-6}M BAP	embryoids	Bhalla-Sarin and Bagga, 1983
Anther	4×10^{-6}M TIBA	embryo	Monfort, 1985
	10.74×10^{-6}M NAA	embryo	Thanh-Tuyen and De Guzman, 1983

ENDOSPERM CULTURE

Polyploid heterosis has occurred in apples, pears, Citrus and grapes and results in greater vigor, larger fruit size and generally greater yield (Sanford, 1983). In Petunia axillaris triploids were more vigorous and had bigger flowers than the diploid or tetraploid plants (Gupta, 1982). Populus tremuloides triploids were superior for wood production compared to diploid counterparts (Johri et al., 1980).

Triploid plants have been produced by crossing diploid and tetraploid plants in Citrus (Soost and Cameron, 1980; Oiyama and Kobayashi, 1990; Oiyama et al. 1991) and in papaya (De Zerpa, 1957) but seed germination was very poor. Survival was low due to the failure of endosperm development and subsequent abortion of embryo which correlates with the ploidy ratio of endosperm to embryo that is not exact 3 : 2 as in vivo (Sita, 1987; Soost, 1987; Grosser, 1994).

Producing triploid plants by crossing method is cumbersome since it takes a long time for economical purpose; an alternative is to regenerate plants from endosperm explants in vitro (Sita, 1987).

Endosperm is triploid in over 81% of flowering plants. It is the result of fusion of two polar nuclei of female gametophyte and one of male gametes (Johri et al., 1980). Therefore, it differs genotypically from the embryo in gene-

dosage (Kovoor, 1981). Endosperm is a fairly homogeneous mass of parenchymatous cells and lacks vascular elements (Johri and Bhojwani, 1977; Johri et al., 1980). However, cells can vary in size and ploidy (Abraham and Mathew, 1963).

The function of endosperm is to nurture the embryo during its heterotrophic phase of growth and to provide combustible sources of energy during seed germination (Johri and Bhojwani, 1977; Raghavan, 1986). Endosperm may be consumed entirely by the embryo so that mature seed is called nonendospermous. If it persists in mature seed as a massive tissue, it can store reserve food materials in the form of starch, fat or protein and is called endospermous (Johri and Bhojwani, 1977; Johri et al., 1980; Bhojwani, 1984).

Endosperm of angiosperms can grow and differentiate into organs (Nag and Johri, 1971; Bhojwani and Razdan, 1983). Triploids from culture may be superior to those from crossing tetraploid and diploid plants, due to the genetically 'unreduced' nature of the 2n polar fusion nucleus in the central cell of the megagametophyte (Knight and Alston, 1969).

Endosperm culture produced shoots or plantlets in Ricinus communis, Exocarpus cupressiformis, Actinidia chinensis, Codiaeum variegatum, Jatropha panduraefolia, Putranjiva roxburghii, Oryza sativa, Dendrophthoe falcata,

Scurrula pulverulenta, Taxillus vestitus, Prunus persica, Pyrus malus, Juglans regia, Citrus grandis, C. sinensis and Santalum album (Rangaswamy and Rao, 1963; Satsangi and Ram, 1965; Johri and Bhojwani, 1977; Bhojwani, 1984; Tulecke et al., 1988; Chen et al. 1990). Thus it may be possible to regenerate coconut plants from cultured endosperm.

The endosperm of coconut is nuclear type with large endospermous seed which endosperm remains until germination (Johri et al., 1992). The endosperm is free of microorganisms (Fernandez, 1988). Even though in vitro culture of adult tissue of coconut is recalcitrant (Reynolds, 1982; D'Souza et al., 1983), regeneration is possible (Branton and Blake, 1983a; 1986; Verdeil et al., 1989). Cultures required high auxin levels for callus initiation, with gradually decreasing auxin concentrations and cytokinin for somatic embryogenesis (SE) induction (Blake, 1983; Verdeil et al., 1994).

FACTORS FOR CULTURE OF COCONUT ENDOSPERM

1. Maturity of explant

The age of the endosperm at the time of culture is critical for growth in vitro (Nag and Johri, 1971). Explants

from younger fruits responded better to culture (Iyer, 1982; Hanower and Pannetier, 1982; Tisserat, 1984; Aitken-Christie et al., 1985; Paranjothy, 1986b; Karunaratne and Periyapperuma, 1989; Karunaratne et al., 1991).

Coenocytic (free-nuclear) endosperm did not survive in culture because of lack of starch (Srivastava, 1982). Endosperm of 6 - 7 months postanthesis in coconut (Kumar et al., 1985), in which solid endosperm started to form in the antipodal end of coconut fruit (Tammes and Whitehead, 1969), was responsive to culture.

Maize endosperm taken between 8 to 12 days after pollination (DAP), grew in vitro when cells were meristematic (Straus and LaRue, 1954; Sternheimer, 1954; Tamaski and Ullstrup, 1958). Endosperm of rye grass responded in culture when excised 9 - 10 DAP (Norstog, 1956), for rice 4 - 7 DAP (Nakano et al., 1975). Mature rice endosperm also gave positive results (Bajaj et al., 1980).

Young endosperm (celliferous stage) was responsive to culture in Citrus grandis and apple (Wang and Chang, 1978; Mu and Liu, 1978). Explants removed 7 - 10 DAP in cucumber (Nakajima, 1962), 56 DAP in walnut (Tulecke, et al., 1988) and 98 - 119 DAP in Citrus sinensis cv. Hongjiang (Chen, et al. 1990) were responsive to culture.

Mature endosperms in Exocarpus cupressiformis, Leptomeria acida, Nigella damascena, Nuytsia floribunda, Osyris wightiana, Scurrula pulverulenta, Phoradendron

tomentosum, Dendrophtoe falcata, Taxillus cunefatus, T. vestitus, Leptomeria acida, Croton bonplandianum, Santalum album, Ricinus communis, Jatropha panduraefolia, Putranjiva roxburghii, Sapium sebiferum, Coffea arabica, Annona squamosa, Achras, apple, parsley and pecan were responsive to culture (Johri and Bhojwani, 1965; Nag and Johri, 1971, Srivastava, 1973; 1982; Sethi and Rangaswamy, 1976; Bajaj et al., 1980; Cheema and Mehra, 1982; Nair et al., 1986, Sita, 1987). Table 2 is a summary of endosperm cultures reported from various plants.

2. Presence or absence of the zygotic embryo

Enclosure of the embryo in the explant was essential for callus initiation in endosperm culture of Dendrophthoe, Exocarpus, Jatropha, Phoradendron, Putranjiva, Scurrula, and Taxillus (Reinert et al., 1977). Mature endosperm of Croton, Ricinus and Putranjiva without enclosed embryos failed to proliferate in vitro (Srivastava, 1982). Mature endosperm of Annona squamosa produced only callus when cultured after radicle emergence 2 - 4 days (Nair et al., 1986). In Citrus sinensis endosperm which had an enclosed embryo for 2 days responded best to culture while failing after 8 days (Chen et al., 1990).

Nag and Johri (1971) reported that an enclosed embryo was not necessary for initiation of callus proliferation of endosperm of 5 parasitic taxa: Dendrophthoe falcata, Nuytsia

Table 2. Endosperm cultures reported from various plants.

Plant	stage	Supplements	Response	Reference
Maize	12 DAP	5000 ppm yeast extract (YE)	callus	Straus and LaRue, 1954
	10-12 DAP	20% clear tomato juice	callus	Sternheimer, 1954
	8-11 DAP	5000 ppm YE	callus	Tamaski and Ullstrup, 1958
	10-12 DAP	9.05×10^{-6}M 2,4-D	callus	Zhu et al., 1988
		without	plantlets	
Rye grass	9-10 DAP	0.25-0.50% YE + 5.71×10^{-6}M IAA	callus	Norstog, 1956
Cucumber	7-10 DAP	5000 ppm YE	callus	Nakajima, 1962
Exocarpus cupressiformis	mature	5.71×10^{-6}M IAA + 4.65×10^{-6}M KN + 400 ppm casein hydrolysate (CH)	callus, shoot or root	Johri and Bhojwani, 1965
Osyris wightiana	mature	22.62×10^{-6}M 2,4-D + 500 ppm CH	callus	Johri and Bhojwani, 1965
		11.42×10^{-6}M IAA + 23.23×10^{-6}M KN	vascularized outgrowth	
Putranjiva roxburghii	mature	9.05×10^{-6}M 2,4-D + 23.23×10^{-6}M KN + 2500 ppm YE	callus	Johri and Bhojwani, 1965
		11.42×10^{-6}M IAA + 23.23×10^{-6}M KN + 1000 ppm CH	callus	Srivastava, 1973
		1.2×10^{-5}M IAA + 2.4×10^{-5}M 2iP	shoots	

Table 2. Endosperm cultures reported from various plants (continued).

Plant	stage	Supplements	Response	Reference
Nuytsia floribunda	mature	24.60×10^{-6}M IBA + 23.23×10^{-6}M KN + 2000 ppm CH	callus	Nag and Johri, 1971
		14.27×10^{-6}M IAA + 23.23×10^{-6}M KN + 2000 ppm CH	tracheid-like cells	
Dendrophthoe falcata	mature	24.60×10^{-6}M IBA + 23.23×10^{-6}M KN + 2000 ppm CH	callus	Nag and Johri, 1971
		14.27×10^{-6}M IAA + 23.23×10^{-6}M KN + 2000 ppm CH	shoots and haustoria	
Taxillus cuneatus	mature	24.60×10^{-6}M IBA + 23.23×10^{-6}M KN + 2000 ppm CH	callus	Nag and Johri, 1971
		14.27×10^{-6}M IAA + 23.23×10^{-6}M KN + 2000 ppm CH	shoot buds	
Leptomeria acida	mature	24.60×10^{-6}M IBA + 23.23×10^{-6}M KN + 2000 ppm CH	callus	Nag and Johri, 1971
		14.27×10^{-6}M IAA + 23.23×10^{-6}M KN + 2000 ppm CH	shoots	
Ricinus communis	mature	22.62×10^{-6}M 2,4-D + 9.29×10^{-6}M KN	callus	Satsangi and Ram, 1965
		22.62×10^{-6}M 2,4-D + 9.29×10^{-6}M KN + 2500 ppm YE11.42×10^{-6}M	embryoids	
		IAA + 23.23×10^{-6}M KN + 1000 ppm CH or 9.05×10^{-6}M 2,4-D + 23.23×10^{-6}M KN + 2500 ppm YE	callus with tracheidal cells and vascular bundle-like	Johri and Srivastava, 1972

Table 2. Endosperm cultures reported from various plants (continued).

Plant	stage	Supplements	Response	Reference
Croton bonplandianum	mature	9.05×10^{-6}M 2,4-D + 23.23×10^{-6}M KN + 2500 ppm YE	callus	Bhojwani and Johri, 1971
		10^{-7}M IAA, IBA or NAA	roots	
Jatropha panduraefolia	mature	9.05×10^{-6}M 2,4-D + 23.23×10^{-6}M KN + 2500 ppm YE	callus	Johri and Bhojwani, 1965
		9.05×10^{-6}M 2,4-D + 23.23×10^{-6}M KN + 2500 ppm YE	callus	Srivastava, 1971
		0.54×10^{-6}M NAA + 2.32×10^{-6}M KN + 500 ppm CH	shoots and roots	
Scurrula pulverulenta	mature	5.71×10^{-6}M IAA + 400 ppm CH	callus	Bhojwani and Johri, 1970
		5.71×10^{-6}M IAA + 4.65×10^{-6}M KN + 400 ppm CH	shoot and haustoria	
Taxillus vestitus	mature	2×10^{-5}M IAA, IBA, NAA, 2,4-D or 2,4,5-T	callus	Johri and Nag, 1970
		2×10^{-5}M KN, BAP or 2iP	shoot buds	
		24.60×10^{-6}M IBA + 23.23×10^{-6}M KN + 2000 ppm CH	callus	Nag and Johri, 1971
		14.27×10^{-6}M IAA + 23.23×10^{-6}M KN + 2000 ppm CH	shoots and haustoria	
Nigella damascena	mature	5×10^{-6}M-10^{-5}M 2,4-D	callus and embryoids	Sethi and Rangaswamy, 1976

Table 2. Endosperm cultures reported from various plants (continued).

Plant	stage	Supplements	Response	Reference
Parsley	mature	without	callus, shoot and root	Masuda et al., 1977
Citrus grandis cv. Bei-pei and C. sinensis cv. Chin-cheng	cellular stage	9.05×10^{-6}M 2,4-D + 22.19×10^{-6}M BAP + 1000 ppm CH	callus	Wang and Chang, 1978
		$5.77\text{-}43.32 \times 10^{-6}$M GA	embryoids and plantlets	
C. sinensis cv. Hongjiang	98-119 DAP	2.26×10^{-6}M 2,4-D + 2.22×10^{-6}M BAP + 1000 ppm CH	callus	Chen et al., 1990
		$0.29\text{-}14.43 \times 10^{-6}$M GA	root and shoot	
C. sinensis cv. Ridge Pineapple	84-98 DAP	9.05×10^{-6}M 2,4-D + 22.19×10^{-6}M BAP + 23.23×10^{-6}M KN + 1000 ppm CH + 500 ppm ME	callus	Gmitter et al., 1990
		1.11×10^{-6}M BAP + 14.8×10^{-6}M Ad + 5.77×10^{-6}M GA + 500 ppm CH	embryos, root or shoot	
C. grandis cv. White Siamese	84-98 DAP	same medium	callus and embryoids	Gmitter et al., 1990
and C. Xparadisi cv. Duncan	84-98 DAP	same medium	callus	Gmitter et al., 1990

Table 2. Endosperm cultures reported from various plants (continued).

Plant	stage	Supplements	Response	Reference
Sandalwood	0.6-0.8 cm green fruit	4.52-9.05x10^{-6}M 2,4-D or + 2.22-8.87x10^{-6}M BAP + 5.37x10^{-6}M NAA	callus	Sita et al., 1980
		5.71x10^{-6}M IAA + 1.33x10^{-6}M BAP + 1.39x10^{-6}M KN + 2.89x10^{-6}M GA	embryoids	
		2.85x10^{-6}M IAA	shoot and root	
Walnut	young and mature	2,4-D + 4.65x10^{-6}M KN + 500 ppm CH	callus	Cheema and Mehra, 1982
		1.07x10^{-6}M NAA + 2000 ppm CH	roots	
		without	shootbud-like	
cv. Manregian	56 DAP	0.06x10^{-6}M IBA + 4.44x10^{-6}M BAP + 9.29x10^{-6}M KN	embryos	Tulecke et al., 1988
		without	shoot and root	
Pecan cv. Mahan	mature	2,4-D + 4.65x10^{-6}M KN + 500 ppm CH	callus	Cheema and Mehra, 1982
		1.07x10^{-6}M NAA + 2000 ppm CH	roots	
Pear cv. Jinfeng	cellular stage	2.26x10^{-6}M 2,4-D + 2.85x10^{-6}M IAA + 6.66x10^{-6}M BAP	callus	Zhao, 1988
		8.87x10^{-6}M BAP + 28.89x10^{-6}M GA	shoots	
		8.57x10^{-6}M IAA	shoot + root	

Table 2. Endosperm cultures reported from various plants (continued).

Plant	stage	Supplements	Response	Reference
Rice	4-7 DAP	10^{-5}M 2,4-D + 0.4% YE or + $5x10^{-5}$M KN	callus	Nakano et al., 1975
		10^{-5}M IAA + 0.4% YE or $5x10^{-5}$M KN + + 0.4% YE	shoot buds	
	4-8 DAP and mature	$9.05x10^{-6}$M 2,4-D	callus	Bajaj et al., 1980
		$22.84x10^{-6}$M IAA + $9.29x10^{-6}$M KN	shoots and haustoria	
tomato	21 DAP-mature	$4.52x10^{-6}$M 2,4-D + $0.44x10^{-6}$M BAP + $28.89x10^{-6}$M GA	callus	Kagan-Zur et al., 1990
Apple cv. King-kuang	young	$2.26x10^{-6}$M 2,4-D + $4.44x10^{-6}$M BAP + 500 ppm CH	callus	Mu and Liu, 1978
Annona squamosa	mature	$5.37x10^{-6}$M NAA+ $0.88x10^{-6}$M BAP + $0.46x10^{-6}$M KN + $2.89x10^{-6}$M GA	callus	Nair et al., 1986
		$28.54x10^{-6}$M IAA	root	
		$2.69x10^{-6}$M NAA + $8.87x10^{-6}$M BAP	shoot	
Loquat	35-60 DAP	2,4-D, NAA and BAP	callus and embryoids	Chen et al., 1988a
		NAA and Zeatin	plantlets	

Table 2. Endosperm cultures reported from various plants (continued).

Plant	stage	Supplements	Response	Reference
Lycium barbarum	20 DAP	0.45×10^{-6}M 2,4-D	callus	Wang et al., 1988
		0.88×10^{-6}M BAP	plantlets	
Actinidia chinensis	cellular stage	2.26×10^{-6}M 2,4-D or 0.89×10^{-6}M NAA + 13.68×10^{-6}M Zeatin	callus	Gui et al., 1988
		4.56×10^{-6}M Zeatin	plantlets or buds	
Actinidia interspecific hybrids	101-128 DAP	13.7×10^{-6}M Zeatin + 0.54×10^{-6}M NAA + 400 ppm CH	callus and buds	Mu et al., 1990
		23×10^{-6}M Zeatin + 1.7×10^{-6}M IAA + 400 ppm CH	plantlets	

floribunda, Taxillus cuneatus, T. vestitus and Leptomeria acida. Sita (1987) successfully cultured endosperm from fresh green fruits of sandalwood with or without embryos. Proliferation in endosperm cultures of Actinidia (Mu et al., 1990), Citrus grandis, C. sinensis (Wang and Chang, 1978), pear (Zhao, 1988), pecan and walnut (Cheema and Mehra, 1982) occurred without enclosed embryo.

In Croton bonplandianum, Putranjiva and Citrus, the requirement for enclosed embryo was replaced by soaking endosperm pieces in 1 - 2 $mg \cdot l^{-1}$ gibberellic acid (GA) (Johri and Bhojwani, 1977; Srivastava, 1982; Nair et al., 1986) or 1 $mg \cdot l^{-1}$ zeatin (Sita, 1987).

Fisher and Tsai (1978) successfully produced callus from a single explant of young coconut cv. Golden Malayan Dwarf endosperm without any indication of an enclosed embryo. Bhalla-Sarin and Bagga (1983) failed to induce callogenesis of 8 - 12 month old endosperm of coconut cv. West Coast Tall, Dwarf and Laccadive with enclosed embryo. Kumar et al. (1985) were able to induce a subculturable callus from 6 - 7 month old endosperm of coconut cv. West Coast Tall with enclosed embryos.

3. Culture medium

White (W) or Murashige and Skoog (MS) media are commonly used and MS medium is more frequently used because

it contains higher content of inorganic salts and nitrogen (Sharp et al., 1980; Bhojwani, 1984; Chen et al., 1988).

Eeuwens (1976) reported that a new mineral formulation (Y3) was better than W or MS medium for micropropagation of coconut due to mineral deficiencies in macro elements such as nitrogen (ammonium), potassium, phosphate and in micro elements such as iron, iodine, molybdenum in W medium and MS is deficient in iodine. The addition of glutamine, arginine, asparagine and sucrose to Y3 medium increased fresh and dry weight of coconut callus (Eeuwens, 1978).

Gelrite or phytagel gelling agent is a complex of extracellular polysaccharides produced by bacteria Pseudomonas elodea. It is composed of glucuronic acid, rhamnose and glucose and is exceedingly clear. Phytagel has less free minerals and organic impurities than agar (Kyte, 1987; Bonga and Von Aderkas, 1992; Sigma, 1993).

Tissue growth was better on phytagel than agar due to stimulative substances and lack of impurities in culture of coconut (Sugimura et al., 1988), bamboo (Huang and Murashige, 1983), mango (DeWald et al., 1989a) and cucumber (Ladyman and Girard, 1991). Phytagel (1.7 - 1.75 $g \cdot l^{-1}$) was more effective for proliferation of globular and SE of longan and mango (Litz, 1988; DeWald et al., 1989a; 1989b).

Liquid medium is sometimes best for certain culture stages. Liquid static medium was better than agar for initiating coconut callus (Blake and Eeuwens, 1982). Stirred

liquid medium improved the growth rate of oil palm callus (Lioret, 1982).

Age of medium containing 2,4-D influenced growth response of coconut cultures. Explants showed minimal growth and died on one day-old medium, while a better response occurred with five day-old medium and the best response on nine day-old medium due to 2,4-D concentration (Ebert and Taylor, 1990).

4. Growth regulators

Whole plants are autonomous with regards to growth regulators but isolated tissues or cells require auxins or cytokinins to initiate and maintain growth until they become habituated or organized (Everett, et., 1978). Morphogenesis in vitro can be regulated by growth regulators (Christianson and Warnick, 1983). Skoog and Miller (1957) found that the balance of auxin and cytokinin in culture medium governed organogenesis. Auxins induced callogenesis, adventitious plantlets and roots in palms (Tisserat, 1979a; Paranjothy and Rohani, 1982; Reynolds, 1982; Paranjothy, 1986a; 1987).

Among auxins, 2,4-D was the most effective when compared to indole acetic acid (IAA), 2-naphthoxy acetic acid (NOA), α-naphthalene acetic acid (NAA), or indole butyric acid (IBA) in coconut culture (Blake and Eeuwens, 1982; Pannetier and Buffard-Morel, 1986; Karunaratne and Periyapperuma, 1989). Bhalla-Sarin et al. (1986) reported

that IAA-conjugates (IAA-asp and IAA-ala) induced callus and differentiation of coconut embryos.

Auxin at high concentrations (10^{-5}M - 10^{-3}M) was necessary for callus induction in palm, especially on medium supplemented with 1 - 3 $g \cdot l^{-1}$ activated charcoal (AC) (Karunaratne and Periyapperuma, 1989; Branton and Blake, 1983a; Sugimura and Salvana, 1989; Tisserat and DeMason, 1980; Kumar et al., 1985; Jesty and Francis, 1992; Reynolds and Murashige, 1979; Sharma et al., 1984; Zaid and Tisserat, 1984; Gupta et al., 1984; Blake and Eeuwens, 1982).

For mass propagation, somatic embryogenesis (SE) is preferable to organogenesis since it produces integral shoot and root meristems, a large number of plants, and it is also better for genetic engineering and encapsulation as artificial seeds (Gupta, 1987; Sita, 1987; Chen et al., 1988).

Embryogenesis begins in callus cultures with a single cell which differs from neighbouring vacuolated parenchymatous cells by having dense cytoplasm, starch accumulation and it is often surrounded by a thickened cell wall. Internal divisions of the small densely cytoplasmic cells form proembryos and can lead to the development of globular, heart shaped and torpedo shaped embryos which can be converted into plants (Kohlenbach, 1978).

Undefined primordia are initiated in disorganized callus. These may be shoot and/ or root primordia or even

suppressed somatic embryos ["proembryogenic masses" (PEM)] (Wernicke and Milkovits, 1986).

Manipulating the concentration of hormones resulted in SE maturation (Bhaskaran, 1985). 2,4-D is usually essential for culture establishment but is usually removed or its concentration is lowered for growth and differentiation (Murashige, 1974). Low concentrations of "weak" auxins such as NAA or IBA together with low concentrations of cytokinins such as BAP or KN promoted shoot development of oil palm embryos, while roots were obtained in liquid media after brief exposure to NAA (Paranjothy and Rohani, 1982).

The use of auxins other than 2,4-D can overcome the failure of some callus cultures to differentiate due to the presence of residual 2,4-D (Murashige, 1974; Brown and Charlwood, 1990). Another auxin with properties similar to 2,4-D is 4-amino-3,5,6-trichloropicolinic acid (picloram). Picloram promoted the growth of wheat, oat, pea, and tomato explants in vitro (Kefford and Caso, 1966).

Picloram has been successfully applied for callogenesis in date palm (Omar and Novak, 1990) and embryogenesis in pejibaje palm (Valverde et al., 1987). Other plants have also responded to picloram including the following: bamboo (Huang and Murashige, 1983), sugarcane (Fitch et al., 1983; Fitch and Moore, 1990), Psoralea bituminosa, Nicotiana tabacum, Salvia mellifera (Goodin and Becher, 1987), Gasteria and Haworthia (Beyl and Sharma, 1983).

In hybrid sugarcane, picloram maintained regeneration ability for more than 12 months, whereas 2,4-D did not (Fitch and Moore, 1990). Picloram was faster than 2,4-D for callogenesis, embryo induction, and final yield of embryos in Gasteria and Haworthia (Beyl and Sharma, 1983). On the other hand, Saccharum spontaneum clones were slower to form callus (Fitch et al., 1983). More phenolics were produced from cut surfaces of Saccharum spontaneum on picloram compared to 2,4-D. Also some sugarcane hybrids produced callus sooner on 2,4-D than on picloram. However, picloram was utilized to maintain regenerative callus lines over long periods of time (Fitch et al., 1983).

Plant tissues release peroxidases and IAA oxidases into medium. These degrade IAA. Conjugates of IAA protect it from peroxidases and later liberate IAA for hormonal regulation of cell activity (Cohen and Bandurski, 1978; Bonga and Von Aderkas, 1992). IAA-ala or IAA-asp induced callogenesis and IAA-asp with KN induced plant regeneration of coconut embryos (Bhalla-Sarin et al., 1986). IAA-ala stimulated callus growth, root initiation and inhibited shoots, whereas IAA-asp stimulated shoot number in tomato leaf culture (Pence and Caruso, 1984).

The presence of cytokinins, auxins and high concentrations of sucrose (0.2 M) stimulated growth of coconut and date callus (Eeuwens, 1978). Cytokinin at low concentration together with high concentrations of auxins were necessary

for callogenesis and cytokinin at high concentration was necessary for growth of coconut embryos (Bhaskaran, 1985). However, exogenous cytokinin was unnecessary for initiation of callogenesis of bamboo species (Huang and Murashige, 1983). Zeatin promoted embryogenesis of carrot cell but 6-benzylaminopurine (BAP) and kinetin (KN) were inhibitory (Fujimura and Komamine, 1975).

The role of cytokinins was uncertain in palm regeneration (Brackpool et al., 1986). Low concentration of auxin and KN with high concentration of casein hydrolysate (CH) and sucrose favored differentiation of embryoids in oil palm (Cui et al., 1984). KN alone was more effective in inducing differentiation than in combination with IAA and in the absence of KN callus failed to differentiate since IAA inhibited the effect of KN (Johri and Bhojwani, 1977).

Srinivasan et al. (1985) successfully induced SE of Christmas palm with 5 - $50x10^{-6}$M 2,4-D and $5x10^{-6}$M BAP, and plantlet formation occurred on hormone-free medium with glutamine. However, auxin or cytokinin inhibited SE in 'Shamouti' orange cultures (Kochba and Spegel-Roy, 1977).

Cytokinins were not necessary for embryogenesis in most graminous species but might be used in conjunction with 2,4-D for induction and maintenance of embryogenic calli (Vasil and Vasil, 1984). Callus was initiated with either BAP or KN while 2,4-D alone was not effective in longan, BAP was much less effective for induction of embryogenic competency than

KN, although it was more effective in stimulating callus growth (Litz, 1988).

Some cytokinins were important in rapid development of embryoids in oil palm leaf callus (Pannetier et al., 1981). BAP greatly increased the fresh weight of coconut callus (Eeuwens, 1978; Kuruvinashetti and Iyer, 1980) and date palm callus (Eeuwens, 1978; Sharma et al., 1984). Among cytokinins, 6-(γ-γ-dimethylallylamino) purine (2iP) was most active when compared to BAP or KN (Murashige, 1974).

Adenine (Ad) enhanced organ initiation and favored multiplication of diploid cells over endopolyploid cells (Murashige, 1974). Adenine sulfate (AdS) at 25 $mg \cdot l^{-1}$ improved plantlet growth of oil palm (Thomas and Rao, 1985). Zeatin was the most effective for shoot development of Actinidia chinensis (Harada, 1975), and at a level of 0.5 $mg \cdot l^{-1}$ increased the number of buds in date culture (Branton and Blake, 1989).

KN at $2x10^{-5}M$ was the most effective cytokinin in producing shoot buds with Taxillus vestitus endosperm (Johri and Nag, 1970). Other researchers used a combination of two cytokinins, such as KN and BAP on culture of sandalwood endosperms (Sita et al., 1980), Annona squamosa (Nair et al., 1986), coconut embryos (Gupta et al., 1984; Karunaratne and Periyapperuma, 1989) or three cytokinins, such as AdS, 2iP and BAP on coconut inflorescences (Branton and Blake, 1983a; 1986).

After treatment with high auxin levels, transfer to hormone free medium or medium with very low auxin concentration induced embryo germination of callus in oil palm leaves (Rao et al., 1987), Christmas palm embryos (Srinivasan et al., 1985), date palm embryos (Reynolds and Murashige, 1979; Zaid and Tisserat, 1984), date palm axillary buds (Tisserat and DeMason, 1980; Sharma et al., 1984), date palm shoot tips (Sharma et al., 1984), Brachea dulchis, Livistona decipiens, Phoenix pusilla, P. sylvestris, Prostoea sp. and Sabal minor embryos (Zaid and Tisserat, 1984), Howeia fosteriana and Chamaedorea costaricana embryos (Reynolds and Murashige, 1979) and Euterpe edulis embryos (Guerra and Handro, 1988).

In coconut, auxins, particularly 2,4-D were found to be essential for embryogenesis, as complete withdrawal of 2,4-D suppressed embryogenesis (Brackpool et al., 1986; Karunaratne and Periyapperuma, 1989). 2,4-D at 12 - 20x10^{-6}M induced embryogenic callus of 6 - 7 month-old embryos (diameter 0.2 - 1.5 mm) and plant regeneration occurred when BAP and KN at 10^{-5}M each were incorporated into the medium containing 2 - 8x10^{-6}M 2,4-D (Karunaratne and Periyapperuma, 1989).

Combination of 2,4-D and BAP induced SE of young and mature coconut leaf (Pannetier and Buffard-Morel, 1982a). Additionally, 10^{-4} M 2,4-D with 5x10^{-6}M BAP, 5x10^{-4}M 2iP and 27x10^{-7}M AdS induced embryoids and plantlets from

inflorescence tissues of coconut cv. Malayan Dwarf (Branton and Blake, 1983a; 1986).

Gibberelic acid (GA_3) was used for germination of somatic embryos in sandalwood (Sita et al., 1980), horse chesnut (Radojevic, 1988) and fostered well-organized shoot and root production of embryonal structures in oil palm callus culture (Nwankwo and Krikorian, 1986).

Antiauxin, i.e. 7-aza-indole (AZI) and 5 hydroxy nitrobenzylbromide (HNB) stimulated embryogenesis in orange cv. Shamouti (Kochba and Spegel-Roy, 1977), whereas B-napthelene acetic acid, phenylpropionic acid, triiodobenzoic acid (TIBA), 2(p-chlorophenoxy) 2-methylpropionic acid (PCMP) as well as abscisic acid (ABA) prevented recallusing of embryoids in soapnut (Sapindus trifoliatus) (Desay et al., 1986).

ABA promoted accumulation of storage lipids, carbohydrates and proteins in somatic embryos (Arnold and Hakman, 1988; Hakman and Arnorld, 1988; Feirer et al., 1989; Roberts et al., 1990), supressed abnormal development, inhibited precocious germinaton and promoted maturation of somatic embryos in conifer cultures (Durzan and Gupta, 1987; Boulay et al., 1988; Roberts et al., 1990).

Putrescine is a polyamine, and can be associated with control of cell growth and division in microbial, animal

and plant systems (Audisio et al., 1976; Fienberg et al., 1984; Feirer et al., 1984; Wu and Kuniyuki, 1985; Evans and Malmberg, 1989). Polyamines stimulate DNA, RNA, and protein synthesis in plants and animals. An increasing concentration of polyamines is associated with intense mitotic activity in meristematic tissues of tomato and potato (Cohen et al., 1982) and the initiation of sprouting potato tubers (Kaur-Sawhney et al., 1982).

Montague et al. (1978) and Bradley et al. (1984) reported that polyamines were involved in cellular differentiation during embryogenesis. Addition of putrescine to the culture medium restored embryogenesis in cultures of wild carrot petiole (Feirer et al., 1984). Putrescine at level 0.1×10^{-6}M - 10^{-3}M was the most effective polyamine for stimulating division of almond protoplast-derived cells (Wu and Kuniyuki, 1985; Evans and Malmberg, 1989) and increased plant regeneration in apple leaf culture (James et al., 1988).

5. Browning

Explant browning is often associated with failure of explant survival. In some adult materials, it may be very severe, especially in palms which causes the inhibition or

cessation of growth (Ventura et al., 1966; Balaga and De Guzman, 1971; De Guzman et al., 1971; Kuruvinashetti and Iyer, 1980; Monfort, 1985; Paranjothy, 1986a, 1987; Krikorian, 1988; Paranjothy et al., 1990). However, explants which have become completely black can provide successful cultures (Jones, 1974).

Phenolics, tannins or oxidized polyphenols are synthesized through shikimic acid, phenylpropanoid, flavonoid and terpenoid pathways. These substances are abundantly present in some plants and act as inhibitory agents (Preece and Compton, 1991). Polyphenol is the most common one (Forrest, 1969; Davies, 1972). Phenolics cause oxidative browning in explants which leads to discoloration of the culture medium. Oxidized phenolic compounds are frequently exuded into the medium by injured woody tissues causing lethal browning or blackening of explants (Alderson, 1987).

Immature endosperms were more responsive in vitro than mature ones (Cheema and Mehra, 1982). This was partially due to oxidation products which were more abundant in the older explants (Sugimura et al., 1988; Preece and Compton, 1991).

Browning can be reduced or eliminated through the use of liquid media, activated charcoal, and anti-oxidants such as ascorbic or citric acids, sodium hydrosulfite, cystein, diethyldithiocarbamate (DTT), potassium ethylxanthate, thiourea, benzimidazole, sodium bisulfite,

polyvinylpyrrolidone (PVP), polyclar, glutathione and bovine serum albumin. Furthermore, more frequent transfers, incubation with reduced illumination or in complete darkness can also reduce browning. Presoaking explants in sterile water, changing medium, avoiding high temperatures, discarding explants which show browning, reducing explant thickness and choosing the most suitable stage for explants are important considerations for the minimization of browning (Murashige, 1974; Reynolds, 1982; Tisserat, 1984; Pannetier and Buffard-Morel, 1986; Sugimura and Salvana, 1989; Monnier, 1990a; 1990b; Preece and Compton, 1991; Jesty and Francis, 1992; Krikorian, 1994).

Addition of auxins, particularly 2,4-D at high concentrations, led to browning of coconut leaf (Pannetier and Buffard-Morel, 1986). Sugimura and Salvana (1989) observed that 2,4-D at 2.26×10^{-4}M - 4.52×10^{-4}M caused severe browning of tissues regardless of the stage and size of coconut inflorescence culture. 2,4-D at levels higher than 30×10^{-6}M inhibited callusing and enhanced browning of coconut embryos (Karunaratne and Periyapperuma, 1989).

Callus precociously isolated from explants also caused browning and necrosis with coconut inflorescences (Verdeil et al., 1994). Addition of cytokinin, i.e. KN also caused more browning than auxin in palm cultures (Reynolds, 1982).

6. Culture period

Callus aging prior to subculture increased embryoid development in Citrus sinensis. Six and 14-week-old callus prior to subculture produced 10 times and 100 times, respectively more than three-week-old callus but 20-week-old callus lost totipotency (Kochba and Button, 1974).

Cells in callus increased in ploidy and lose regeneration potential with prolonged culture (Reinert and Backs, 1968; Johri and Srivastava, 1973; Smith and Street, 1974). In Citrus limon, cells declined from 100% diploid to 71% within one month and to 33% by the end of three months (Murashige et al., 1967).

However, endosperm cultures of rye grass remained triploid after 10 years of culture (Norstog et al., 1969). In endosperm culture of cucumber, there were abnormal nuclear divisions (Nakajima, 1962), a high degree of polyploidization and various kinds of mitotic irregularities, such as chromosome bridges and lagging chromosomes in rye grass, maize, Croton, Jatropha and Lolium (Norstog, 1956; Johri and Bhojwani, 1977).

According to Dutt (1953), the nuclei of coconut endosperms were of varied sizes (diameter from 16.6 to 72.2 μ) and chromosome numbers [32 (2n), 48 (3n), and 160 (10n)], whereas Abraham and Mathew (1963) observed numbers of 48 (3n), 96 (6n), and 192 (12n). The endosperm callus of parsley consisted primarily of triploid cells but plants

derived were predominantly diploid (Masuda et al., 1977).

Regenerating capacity in parsley was retained for one-half year in parsley cultures (Masuda et al., 1977), one year in sandalwood cultures (Sita et al., 1980), two years in Putranjiwa cultures and 30 months in Dendrophthoe and Taxillus cultures (Johri and Srivastava, 1973; Johri and Nag, 1974).

Rapidly growing callus (doubling time of 10 to 15 days) of oil palm retained embryonic potentiality after five years of subculture on auxin-containing media (Hanower and Pannetier, 1982).

CHAPTER III

INITIATION OF CALLOGENESIS

INTRODUCTION

Coconut explants, especially mature plants are recalcitrant *in vitro*. Callus formation (callogenesis) is uncertain, and is influenced by endogenous and exogenous factors such as genotype, physiological maturity, media and disinfestant.

Fisher and Tsai (1978) reported callogenesis occurred in only a single explant of young coconut cv. Golden Malayan Dwarf endosperm *in vitro* on White medium. Kumar et al. (1985) had over 30% callogenesis from endosperm explants with enclosed embryos from 6 - 7 month-old of coconut cv. West Coast Tall on Eeuwens medium. They also limited the use of disinfestant solution. Bhalla-Sarin and Bagga (1983) failed to get callogenesis from 8 - 12 month-old coconut cv. West Coast Tall, Dwarf and Laccadive.

Coconut endosperm is free of microorganisms (Fernandez, 1988) and an axenic explant can be obtained from the inside of a surface sterilized fruit.

Preliminary experiments showed that coconut endosperm at 7 - 8 months postanthesis (spoon stage) has already developed enough cellular endosperm on the antipodal and

micropylar regions, to harvest sufficient explants for experiments. The embryo was not necessary for initiation of callogenesis, and slicing the endosperm inhibited tissue growth. The medium formulation of Branton and Blake (1986) with the addition of putrescine and phytagel produced earlier and faster callus growth than those of Kumar et al. (1985) or Fisher and Tsai (1978).

The objective of this chapter is to establish a protocol for initiation *in vitro* of callogenesis of coconut endosperm.

MATERIALS AND METHODS

Two fruits were taken from one coconut tree (*Cocos nucifera*) cv. Samoan Dwarf grown in the Magoon greenhouse facility of the University of Hawaii at Manoa, on January 26 and January 27, 1994. Another two fruits from different trees grown in Moanalua, Oahu were harvested on April 26, 1994.

Fruits were surface disinfested with 95% ethyl alcohol, punctured to remove liquid endosperm and cut longitudinally with a sterile knife. Solid cylindrical endosperm plugs (8 mm in diameter and 2 - 6 mm thick) were aseptically cored with a cork borer and scooped with a spoon. Explants were taken from either the micropylar region (upper half of fruit

with embryo) or the antipodal region (bottom half of fruit).

The basal medium (BM) of (Branton and Blake, 1986) was modified by addition of 10 $mg \cdot l^{-1}$ putrescine and substitution of agar with 1.7 $mg \cdot l^{-1}$ phytagel (Sigma Chemical Co. p-8169). Activated charcoal (AC) from Sigma Chemical Co. c-4386 was used at 2.5 $g \cdot l^{-1}$ and supplemented with various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) or 4-amino-3,5,6-trichloropicolinic acid (picloram) at 0, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3}M. 6-benzylaminopurine (BAP) was used in some treatments at 10^{-5}M.

The pH was adjusted to 5.7 before it was autoclaved. The medium was poured into 2.5 x 15 cm test tubes (14 ml) or into 125 ml erlenmeyer flasks (40 ml) and autoclaved at 121°C and 1 $kg \cdot cm^{-2}$ pressure for 15 minutes. After autoclaving, the medium was shaken every 10 minutes before gelling in order to disperse AC.

The medium was stored for about one week as recommended by Ebert and Taylor (1990), in order to equilibrate growth regulators in AC. Single explants were placed into test tubes with the uncut surface [(facing away from the endocarp (shell)] upright. Each tube was weighed prior to and after culture. The cultures were incubated at approximately 31°C in the dark.

The experimental design was a randomized complete block design (RCBD) with each fruit as a block. Treatments were

factorial combinations of endosperm region (micropylar and antipodal), auxin (2,4-D and picloram) and their concentrations (0, 10^{-6}M, 10^{-5}M, 10^{-4}M and 10^{-3}M) and cytokinin (with or without 10^{-5}M BAP) with 12 replications.

The schedule for subculture was as follows for initiation of callogenesis, callus growth and morphogenesis:

Auxin [2,4-D (D) or picloram (P)]	Transfer [weeks of culture (WOC)]				
Initial conc.	Trans.1 (9 WOC)	Trans.2 (16 WOC)	Trans.3 (21 WOC)	Trans.4 (26 WOC)	Trans.5 (31 WOC)
0	BM	BM	BM	BM	BM
10^{-6}M D	10^{-6}M D	10^{-7}M D ± 10^{-5}M BAP	10^{-8}M D ± 10^{-5}M BAP	BM	BM
10^{-6}M P	10^{-6}M P	10^{-7}M P ± 10^{-5}M BAP	10^{-8}M P ± 10^{-5}M BAP	BM	BM
10^{-5}M D	10^{-6}M D	10^{-7}M D ± 10^{-5}M BAP	10^{-8}M D ± 10^{-5}M BAP	BM	BM
10^{-5}M P	10^{-6}M P	10^{-7}M P ± 10^{-5}M BAP	10^{-8}M P ± 10^{-5}M BAP	BM	BM
10^{-4}M D	10^{-6}M D	10^{-7}M D ± 10^{-5}M BAP	10^{-8}M D ± 10^{-5}M BAP	BM	BM
10^{-4}M P	10^{-6}M P	10^{-7}M P ± 10^{-5}M BAP	10^{-8}M P ± 10^{-5}M BAP	BM	BM
10^{-3}M D	10^{-6}M D	10^{-7}M D ± 10^{-5}M BAP	10^{-8}M D ± 10^{-5}M BAP	BM	BM
10^{-3}M P	10^{-6}M P	10^{-7}M P ± 10^{-5}M BAP	10^{-8}M P ± 10^{-5}M BAP	BM	BM

Percentage callus formation was computed 31 after WOC. Data were analyzed with the General Linear Models (GLM) procedure of Statistical Analysis System (SAS).

Browning was evaluated visually at every transfer using numerical scores ranging from 0 to 3 (0 = no browning, 1 = little browning, 2 = medium browning, and 3 = high browning). Data were analyzed with GLM and non parametric one way (Kruskal-Wallis test) procedures of SAS.

RESULTS AND DISCUSSION

Explants formed callus after approximately three WOC. Callus appearance varied within the control and was similar to the other treatments (Figure 1). Callus grew predominantly on the uncut surface of the explant and also on the side of the endosperm (Figure 2). Eventually, the callus grew and covered the entire explant. Callus was initially yellow-white and firm, it then became pale in color and slightly friable after 21 WOC (Figure 3). Figure 1 shows the varied growth and development of control explants after four WOC. Some explants remained rather white, brownish and swollen while others turned dark brown and formed callus.

Figure 2 shows initial callus grown from control cultures after 7 WOC. Callus first appeared on top of the explant or from the side which touched the medium.

Figure 3 shows callus which had become pale with friable appearance after 21 WOC. Callogenesis occurred in

almost all explants after 31 WOC, including the control. Average callogenesis was 98.98%, 99.44, 97.92% and 98.98% for fruit 1, 2, 3 and 4, respectively (Table 4).

Callogenesis of antipodal tissues was 98.89%, 98.89%, 97.92% and 100.00% for fruit 1, 2, 3 and 4, respectively (Table 4). Results with micropylar tissues which was 99.07%, 100.00%, 98.15% and 97.96%, respectively (Table 4). There was no significant difference in callogenesis between the two regions of the endosperm.

Callogenesis of tissues treated with 2,4-D was 98.89%, 100.00%, 96.30% and 98.89% for fruit 1, 2, 3 and 4, respectively (Table 4). Callogenesis of tissues treated with picloram which was 99.07%, 98.89%, 99.54% and 99.07% for fruit 1, 2, 3, and 4, respectively (Table 4). There was no significant difference in callogenesis between explants treated with 2,4-D or picloram.

Callogenesis of the control was 100.00%, 100.00%, 97.92% and 100.00% for fruit 1, 2, 3 and 4, respectively (Table 4). Callogenesis of tissues treated with the 10^{-6}M of both auxins was 100.00% for all fruits (Table 4). Callogenesis of tissues treated with the 10^{-5}M of both auxins was 100.00%, 100.00%, 100.00% and 97.92% for fruit 1, 2, 3 and 4, respectively (Table 4). Callogenesis of tissues treated with the 10^{-4}M of both auxins was 97.50%, 100.00%, 95.83% and 100.00% for fruit 1, 2, 3 and 4, respectively (Table 4). Callogenesis of tissues treated with the 10^{-3}M of

both auxins was 97.92%, 97.50%, 95.83% and 97.50% for fruit 1, 2, 3 and 4, respectively (Table 4). Consequently, there was no significant difference in callogenesis between different auxins or their concentrations. Furthermore, auxin did not significantly increase callogenesis compared to the hormone-free control.

Callogenesis of tissues treated with the addition of 10^{-5}M BAP which was 98.96%, 100.00%, 95.83% and 98.96% for fruit 1, 2, 3 and 4, respectively (Table 4). Callogenesis without the addition of BAP was 99.00%, 99.00%, 99.58% and 99% for fruit 1, 2, 3 and 4, respectively (Table 4). Addition of 10^{-5}M BAP did not significantly affect callogenesis.

Tissue (endosperm and callus) browning varied within or among treatments. Figure 4 shows that browning varied from little (I = score 1) to medium (II = score 2) to high (III = score 3) within the control after 21 WOC.

Tissue color changed progressively, from white to tan to brown, to dark brown and to black. Then new yellowish white callus grew from the black callus and this sequence was repeated through many cycles. Figure 5 shows the various colors produced by antipodal tissues treated with 10^{-4}M 2,4-D after 13 months of culture.

Tissue browning by all treatments was slight after 9 WOC (score = 1.18). Tissue browning increased substantially after 16 WOC (score = 1.75) and reached maximal browning

after 21 WOC (score = 2.25). Thereafter browning decreased slightly at 26 (score = 2.23) and 31 WOC (score = 2.18) (Table 6 and Figure 6).

The browning scores of antipodal tissues from all treatments were 1.29, 1.73, 2.27, 2.32 and 2.30 after 9, 16, 21, 26 and 31 WOC, respectively, while those of micropylar tissues were 1.07, 1.76, 2.24, 2.14 and 2.05 after 9, 16, 21, 26 and 31 WOC, respectively (Table 6 and Figure 7). Statistical analysis showed that antipodal tissues had significantly more browning than micropylar tissues at 9, 26 and 31 WOC but there were no significant differences between them at 16 and 21 WOC (Table 6). Thus, the endosperm region had a significant effect on initial browning but not on long term results.

The browning score of tissues treated with 2,4-D were 1.15, 1.73, 2.22, 2.21 and 2.19 after 9, 16, 21, 26 and 31 WOC, respectively, while those treated with picloram were 1.21, 1.77, 2.29, 2.24 and 2.17 after 9, 16, 21, 26 and 31 WOC, respectively (Table 6 and Figure 8). Thus, there was no significant difference in browning between the 2,4-D and picloram treatments.

After 9 WOC, the browning score of the control (1.60) and tissues initially treated with the 10^{-3}M auxin (1.53) were significantly higher than those of the other auxin concentrations (Table 6 and Figure 9).

After 16 WOC, the browning of tissues initially treated

with the 10^{-3}M auxin was the highest while the control was higher than those of tissues treated with other auxins (Table 6 and Figure 10).

After 21 WOC, the tissues initially treated with the 10^{-6}M auxin showed significantly less browning than the control or other auxin concentrations (Table 6 and Figure 11).

After 26 WOC, the tissues initially treated with the 10^{-5}M auxin had a significantly more browning score than the rest of the treatments (Table 6 and Figure 12).

After 31 WOC, the tissues initially treated with the 10^{-3}M auxin had a significantly lower browning score (1.94) than the other treatments (Table 6 and Figure 13). While lower auxin concentrations retarded browning during the initial culture period. Their effect diminished over time. Eventually the highest auxin concentration significantly reduced browning.

The browning scores of tissues treated with 10^{-5}M BAP was 1.43, 2.22, 2.21 and 2.15 on 16, 21, 26 and 31 WOC, respectively, while those without BAP was 1.96, 2.28, 2.24 and 2.20 after 16, 21, 26 and 31 WOC, respectively (Table 6 and Figure 14). Addition of BAP significantly decreased browning after 16 WOC but did not affect it significantly thereafter.

The presence of an embryo in the endosperm explants was clearly unnecessary for callus initiation in coconut. This

contrasts with other report on callogenesis of coconut endosperm (Kumar et al., 1985). Thus, coconut endosperm behaves like endosperms of Actinidia, Croton bonplandianum, Putranjiva, Citrus grandis, Citrus sinensis, pear, pecan, sandalwood and walnut (Johri and Bhojwani, 1977; Wang and Chang, 1978; Cheema and Mehra, 1982; Srivastava, 1982; Nair et al., 1986; Sita, 1987; Zhao, 1988; Mu et al., 1990).

Callus initiation in our studies occurred within three WOC. Similar results occurred with endosperm culture of Exocarpus cupressiformis (Johri and Bhojwani, 1965). However, endosperm culture of Osyris wightiana required 20 weeks to form a callus (Johri and Bhojwani, 1965). Callogenesis occurred after four weeks in coconut endosperm (Kumar et al., 1985), one month in embryo explants of Geonoma gamiova (Dias et al., 1994), while coconut inflorescence cultures required four months for callus initiation (Verdeil et al., 1994). Coconut (Pannetier and Buffard-Morel, 1982b) and oil palm (Hanover and Pannetier, 1982) leaves also required over two months before callogenesis occurred. These disparities could be due to a number of additional factors which include genotype effects, media differences and disinfestation methods. It is important to note that explants were not directly exposed to any harsh chemicals with our disinfestation protocol.

Coconut endosperm callus became pale in color with slightly friable texture. This was similar to fast-growing

oil palm callus (Ahee et al., 1981; Lioret, 1982). In the latter case, this callus morphology was correlated with the development of somatic embryos. In the future, it would be important to make histological observations of coconut endosperm cultures at this stage of callogenesis to see if somatic embryos are present.

The percentage of callogenesis was very high (over 95% for all treatments). Micropylar and antipodal tissues did not differ significantly in callogenesis. This contrasts with Abraham and Mathew (1963) who reported that micropylar region of coconut endosperm was more meristematic than antipodal region. This could have been due to different maturity of fruit and between *in vitro* and *in vivo*.

The growth regulator-free control had very high rates of callogenesis, and auxins did not cause significant differences. However, Karunaratne and Periyapperuma (1989) reported 2,4-D at levels higher than $3x10^{-5}M$ inhibited callogenesis in coconut embryo culture. This could have been due to the use different explants. Results with other embryogenic systems show that high auxin levels can be used for the production of embryogenic callus and complete plants in loblolly pine, especially when high level of activated charcoal (AC) ($2.5\ g \cdot l^{-1}$) are used (Gupta and Durzan, 1987). The AC greatly reduces the effective auxin concentration (Ebert and Taylor, 1990). So it is difficult to make direct comparisons between these studies.

Callogenesis occurred in coconut endosperm without the addition of growth regulators. On the contrary, high auxin concentration was applied for callogenesis in coconut inflorescence cultures (Branton and Blake 1983a; 1986; Sugimura and Salvana, 1989; Verdeil et al., 1994), coconut endosperm cultures (Kumar et al., 1985), date palm axillary bud and shoot tip cultures (Sharma et al., 1984) and oil palm leaf cultures (Thomas and Rao, 1985). This was attributed to endogenous growth regulators in coconut endosperm which is known to contain cytokinins (Zwar et al., 1963; Shaw and Srivastava, 1964; Letham, 1968) and gibberellins (Radley and Dear, 1958). Callogenesis also occurred without any addition of growth regulators in parsley (Masuda et al., 1977) and rice endosperms (Bajaj et al., 1980).

Fisher and Tsai (1978) used coconut endosperm explants of various age and got callus from only one explant but could not repeat these results. This could have been due to the disinfestation method. Bhalla-Sarin and Bagga (1983) failed to induce any callus from endosperm explants probably due to the use of older (8 - 12 month-old) fruits and also their exposure to disinfestants. Whereas Kumar et al. (1985) succeeded in producing over 30% callogenesis in coconut endosperm by swabbed with cotton wool containing 90% ethanol. Our success in obtaining a very high percentage of callus formation is noteworthy and could have been due to

genotypic effects, the use of young explants and their protection from chemical disinfestants as well media differences, including the addition of putrescine and AC.

With other coconut explants, the percentage of callogenesis depended on maturity of the tree. Pannetier and Buffard-Morel (1982b) obtained 50% callogenesis on leaf explants from young trees and only 20% from adult trees; whereas Verdeil et al. (1989) obtained 60% - 70% callogenesis on leaf explants from 5 year-old trees and 30% - 40% on 15 - 20 year old trees. 45% callogenesis was the best obtained from inflorescence explants.

With other plant endosperms, callogenesis occurred 85% in Taxillus vestitus (Nag and Johri, 1971) 29% in Exocarpus cupressiformis (Johri and Bhojwani, 1965), 24.5% in parsley (Masuda et al., 1977), 8 - 25% in grapefruit cv. Duncan (Gmitter et al., 1990) and 1.60 - 3.74% in Citrus sinensis cv. Hongjiang (Chen et al., 1990). Thus our results are in the highest range of successful endosperm callogenesis.

During culture, oxidative browning (brown to black) of explants or calli was often observed. Phenolic compounds exude from excised explants. These compounds are oxidized by peroxidases or polyphenoloxidases, causing browning of both plant tissue and medium (Compton and Preece, 1988). This might reduce growth or kill the tissues (Preece and Compton, 1991).

Severe browning of coconut endosperm occurred in our

cultures even though young explants which were shielded from disinfestants were used. We also incubated our cultures in the dark, as this condition was shown to prevent browning of Geonoma gamiova (Dias et al., 1994).

Explant thickness influenced browning. The antipodal explants which were thicker than micropylar showed more browning. Similar results were obtained by Sugimura and Salvana (1989) with coconut inflorescence explants. Explants 1 mm thick had 32% browning compared to 11% browning with 0.5 mm thick explants.

The two types of auxin did not cause any significant difference in browning of endosperm tissue in this work. Furthermore, high auxin concentrations (10^{-3}M 2,4-D or picloram) did not cause more browning than lower concentrations. On the contrary, Fitch et al. (1983) observed that picloram caused more browning than 2,4-D in Saccharum spontaneum cultures. In addition, Pannetier and Buffard-Morel (1986), Karunaratne and Periyapperuma (1989) or Sugimura and Salvana (1989) observed that 2,4-D levels higher than $3x10^{-5}$M caused more browning in coconut explants than lower concentrations. Concentrations of 2,4-D or picloram at 10^{-4}M or less reduced tissue browning in our work. Similar results were observed by Phillips and Henshaw (1977) on Acer pseudoplatanus cell cultures.

Addition of the cytokinin BAP reduced tissue browning at early stage of callogenesis. Reynolds (1982) reported

that KN caused browning in palm tissues. It might be useful to test other cytokinins to see if they had a moderating effect on browning.

Severe browning did not check further growth of cultures. A similar result was reported by Jones (1974) in oil palm and Ettinger and Preece (1985) in Rhododendron cultures. Coconut endosperm probably tolerated high level of 2,4-D or picloram due to the presence of AC in the medium and due to dark incubation (Wang and Huang, 1976; Friborg et al., 1978; Tisserat, 1979a; Blake and Eeuwens, 1982; Ammirato, 1983; Hu and Wang, 1983; Rao et al., 1987; Sugimura and Salvana, 1989 and Krikorian, 1994).

Fig. 1. Varied growth and development of endosperm after 4 weeks in control. Endosperm tissue was still white, other was brownish and swollen, while others turned dark brown and formed callus.

Fig. 2. Callus first appeared on the top of the explant, another on the side of the explant which touched the medium after 7 weeks in control.

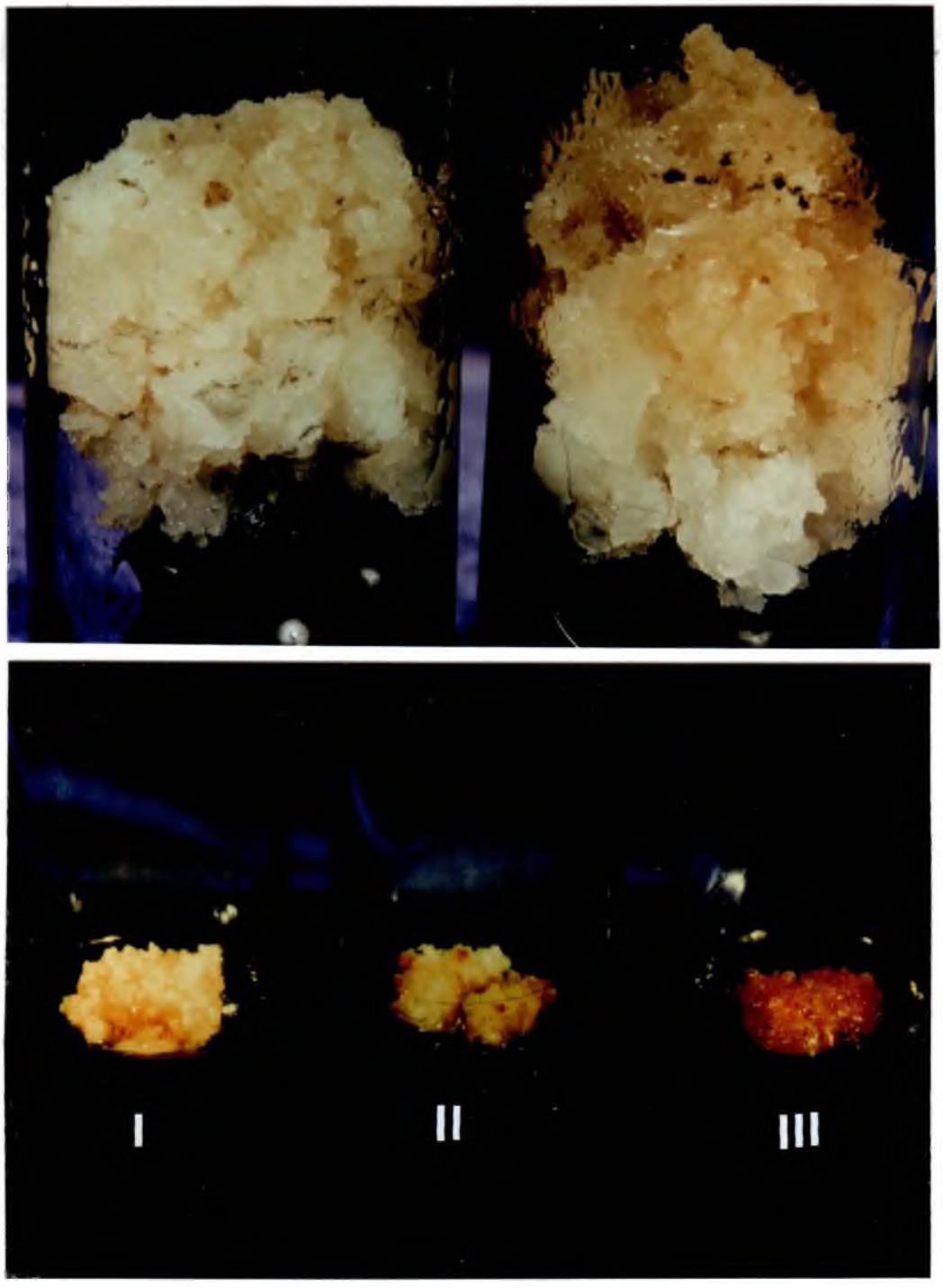


Fig. 3. Callus appearances: pale color and slightly friable after 15 months of culture of tissues treated with 10^{-3}M 2,4-D and another one with addition of 10^{-5}M BAP.

Fig. 4. Tissue (endosperm and callus) browning were varied from little (I = score 1) to medium (II = score 2) and high (III = score 3) in control after 21 weeks.

Fig. 5. Varied colors of callus and new yellowish white callus grown on the black callus of antipodal tissue treated with 10^{-4}M 2,4-D and 10^{-5}M BAP after 13 months.

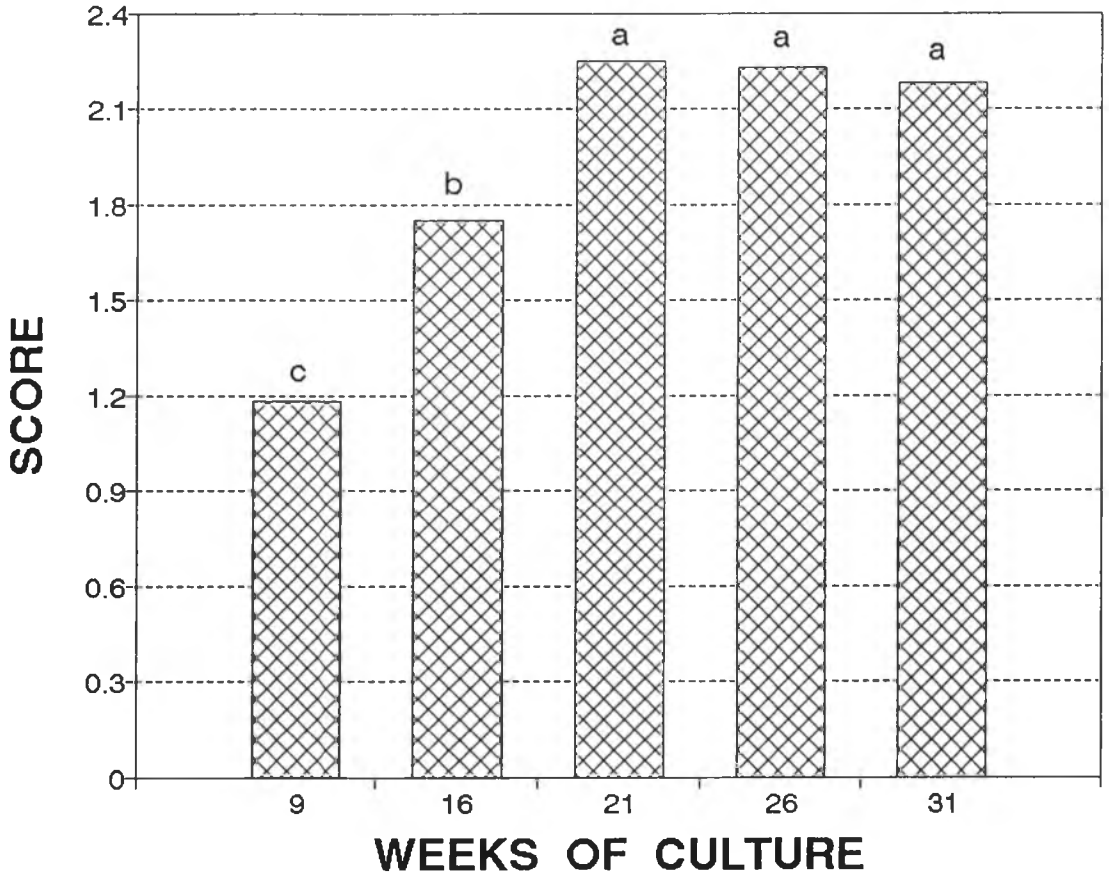


Fig. 6. Average browning score of all treatments (comparison between all possible combinations and same letters are not significant difference at the 5% level).

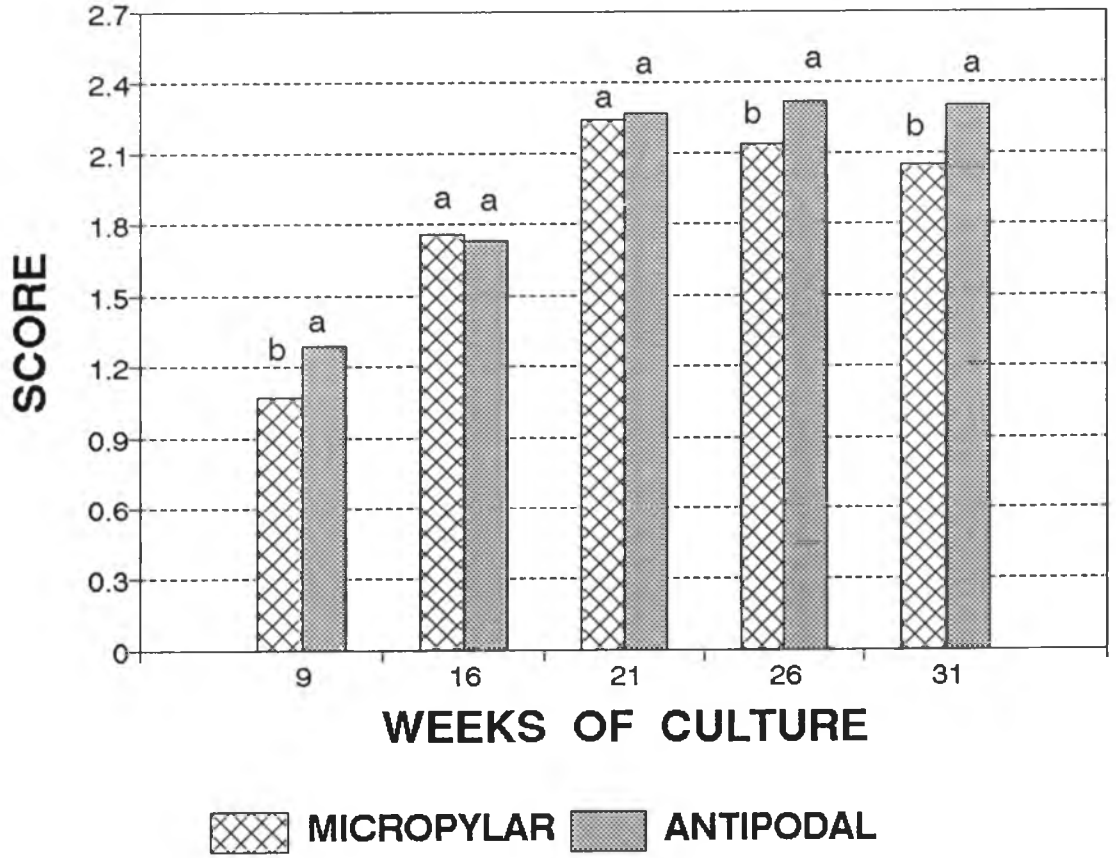


Fig. 7. Effect of endosperm region on tissue browning (same letters are not significant difference at the 5% level).

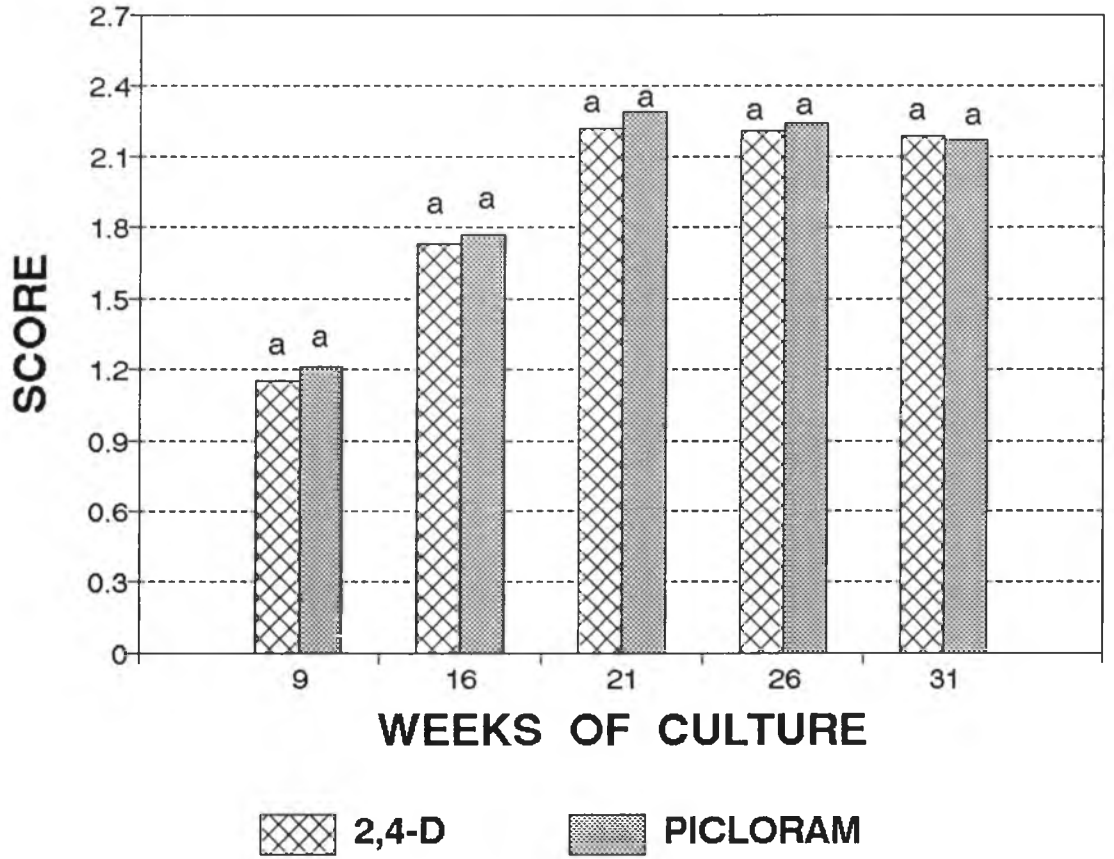


Fig. 8. Effect of 2,4-D and picloram on tissue browning (same letters are not significant difference at the 5% level).

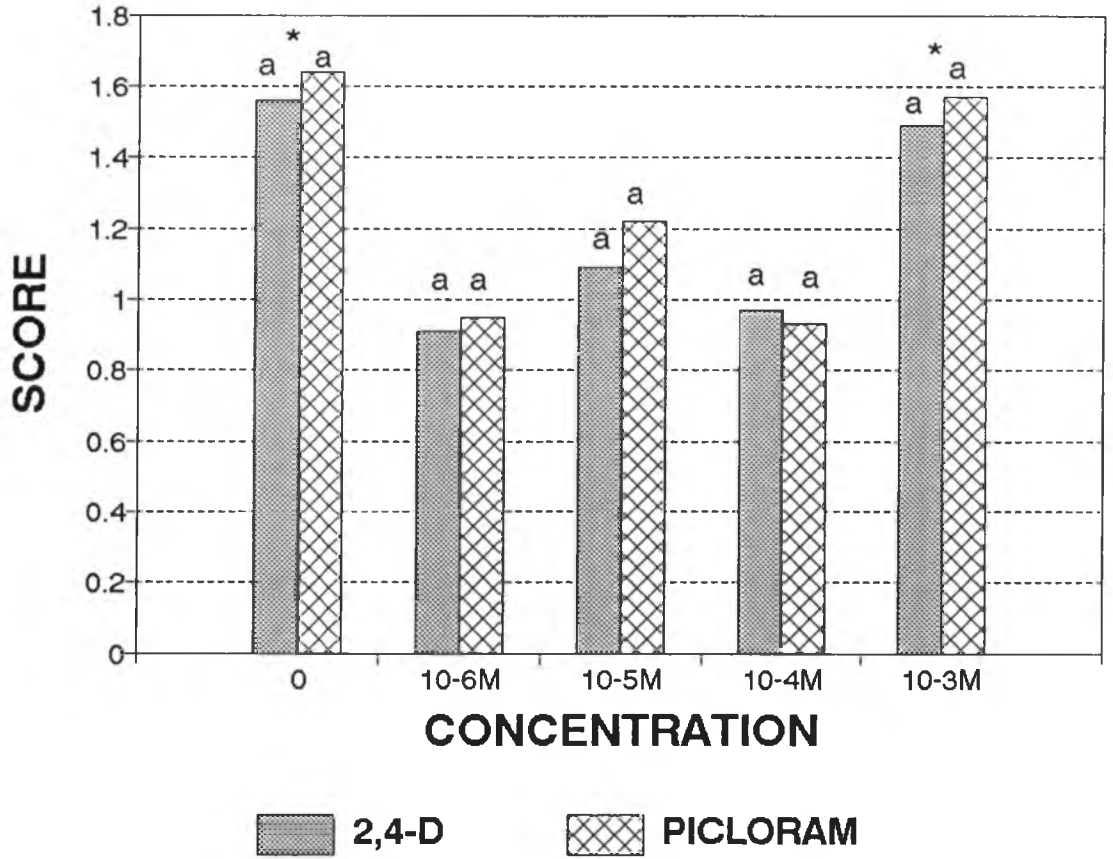


Fig. 9. Effect of 2,4-D and picloram concentrations on tissue browning after 9 weeks of culture (same letters are not significant difference within and * is significant difference among auxin concentrations at the 5% level).

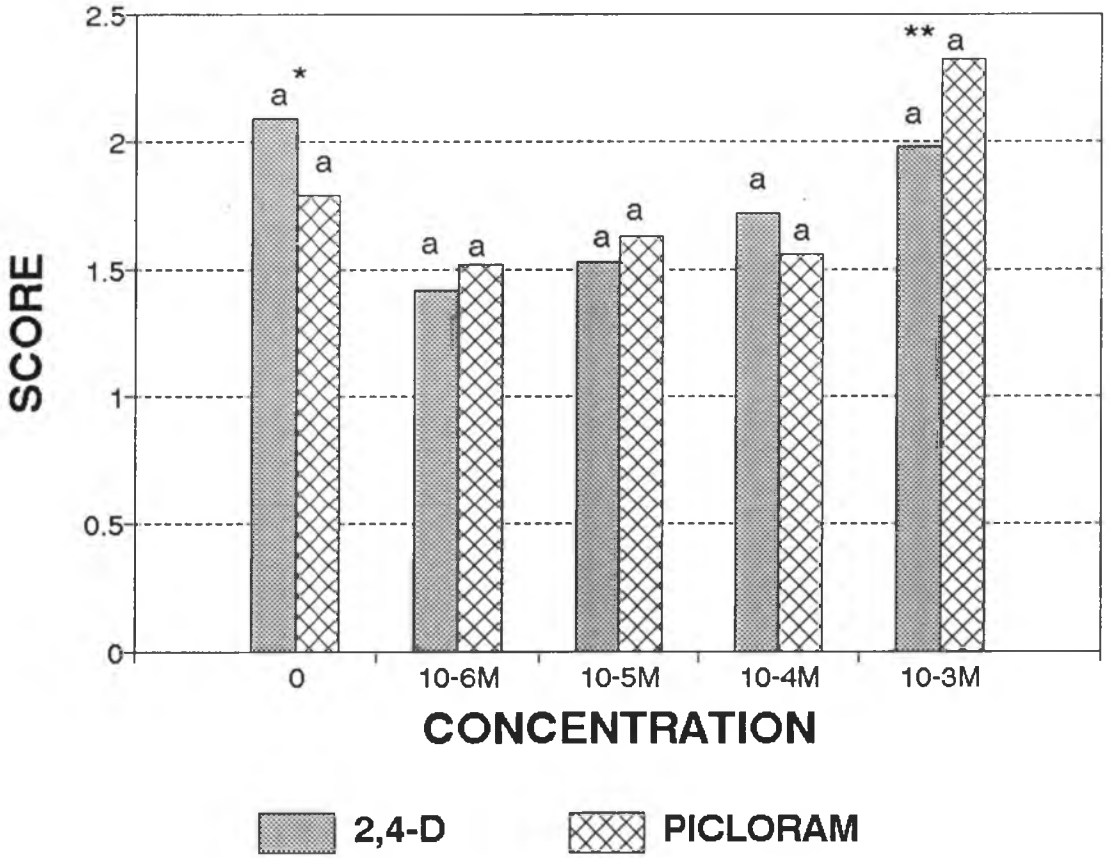


Fig. 10. Effect of 2,4-D and picloram concentrations on tissue browning after 16 weeks of culture (same letters are not significant difference within and * is significant difference among auxin concentrations at the 5% level).

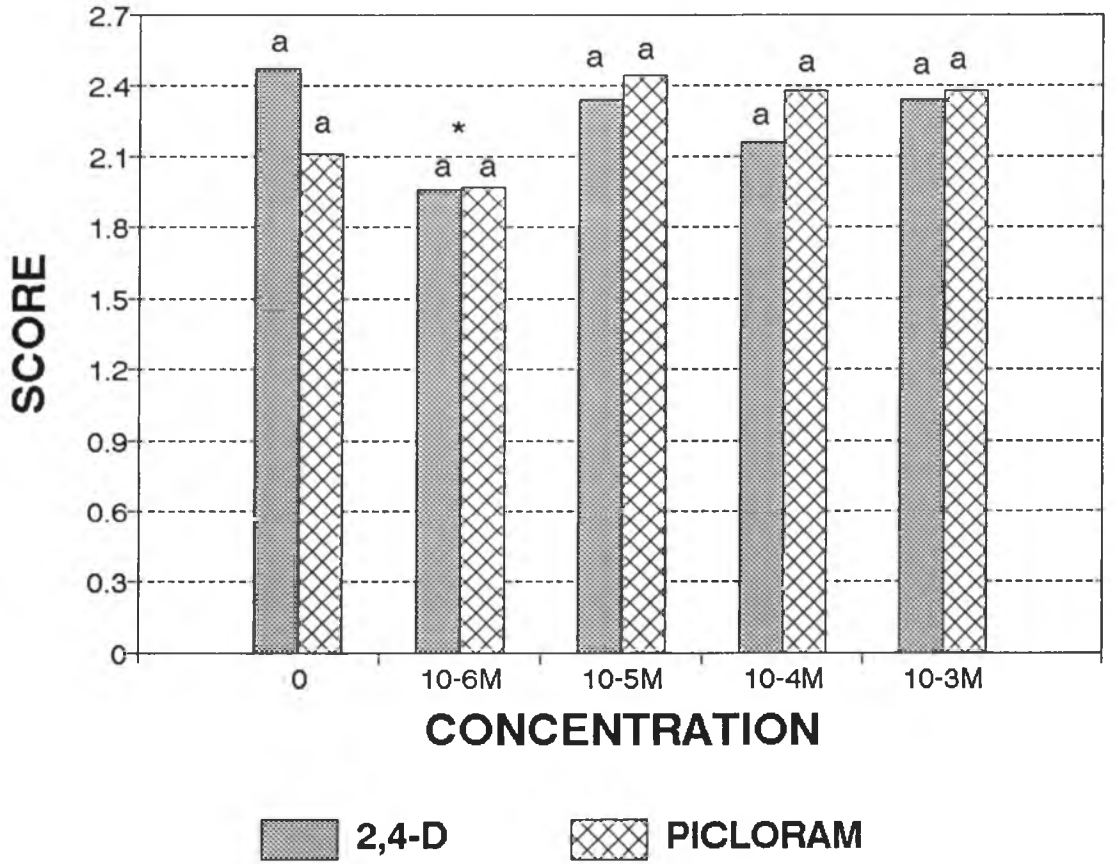


Fig. 11. Effect of 2,4-D and picloram concentrations on tissue browning after 21 weeks of culture (same letters are not significant difference within and * is significant difference among auxin concentrations at the 5% level).

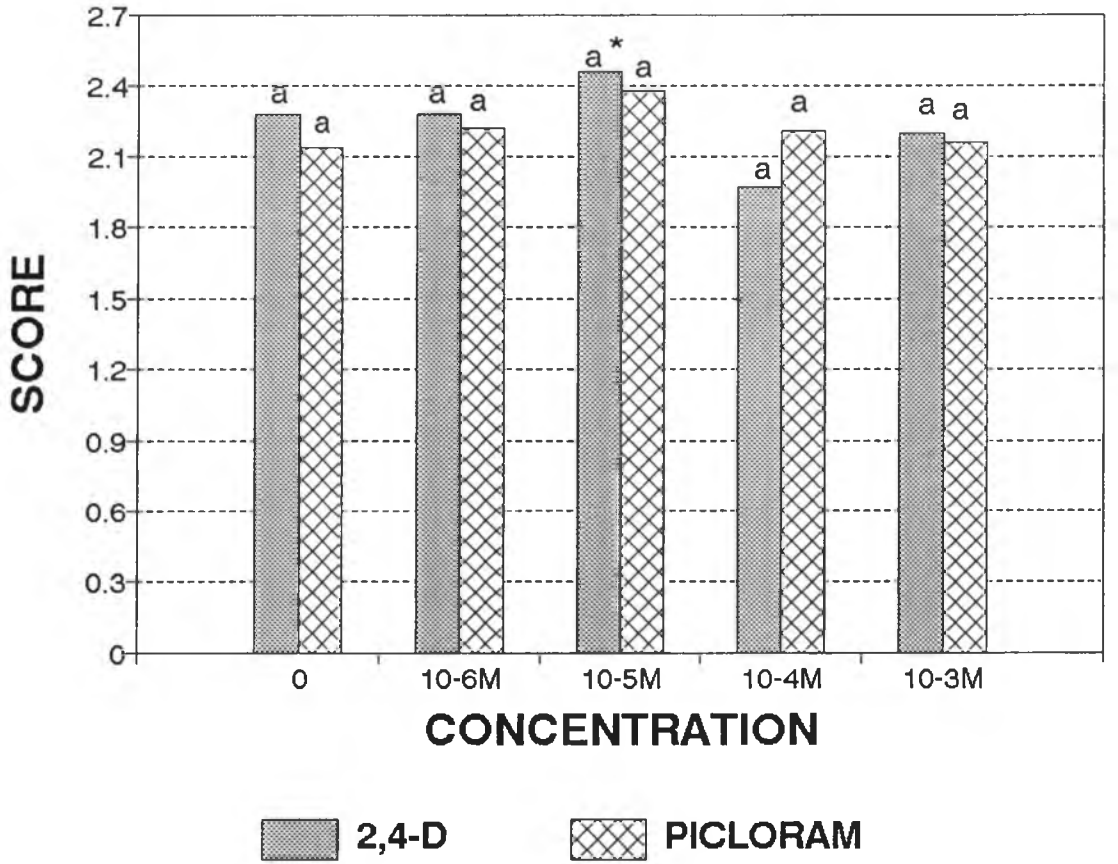


Fig. 12. Effect of 2,4-D and picloram concentrations on tissue browning after 26 weeks of culture (same letters are not significant difference within and * is significant difference among auxin concentrations at the 5% level).

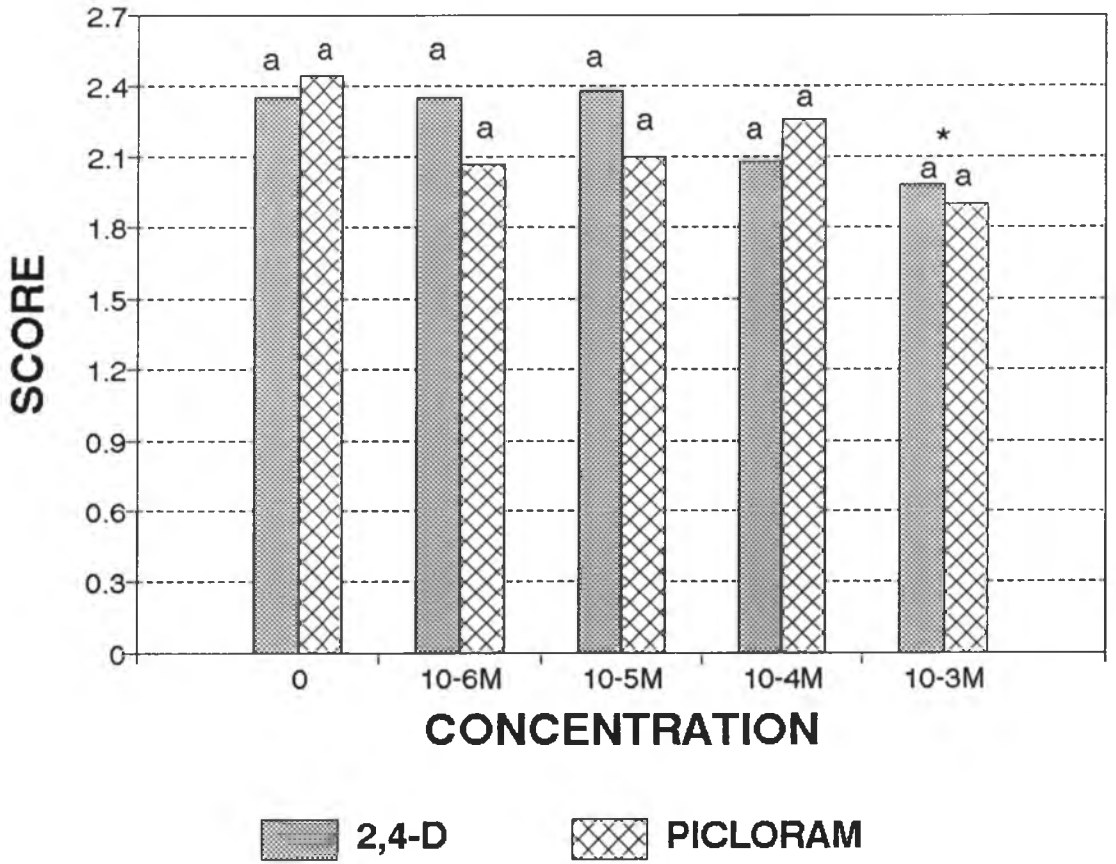


Fig. 13. Effect of 2,4-D and picloram concentrations on tissue browning after 31 weeks of culture (same letters are not significant difference within and * is significant difference among auxin concentrations at the 5% level).

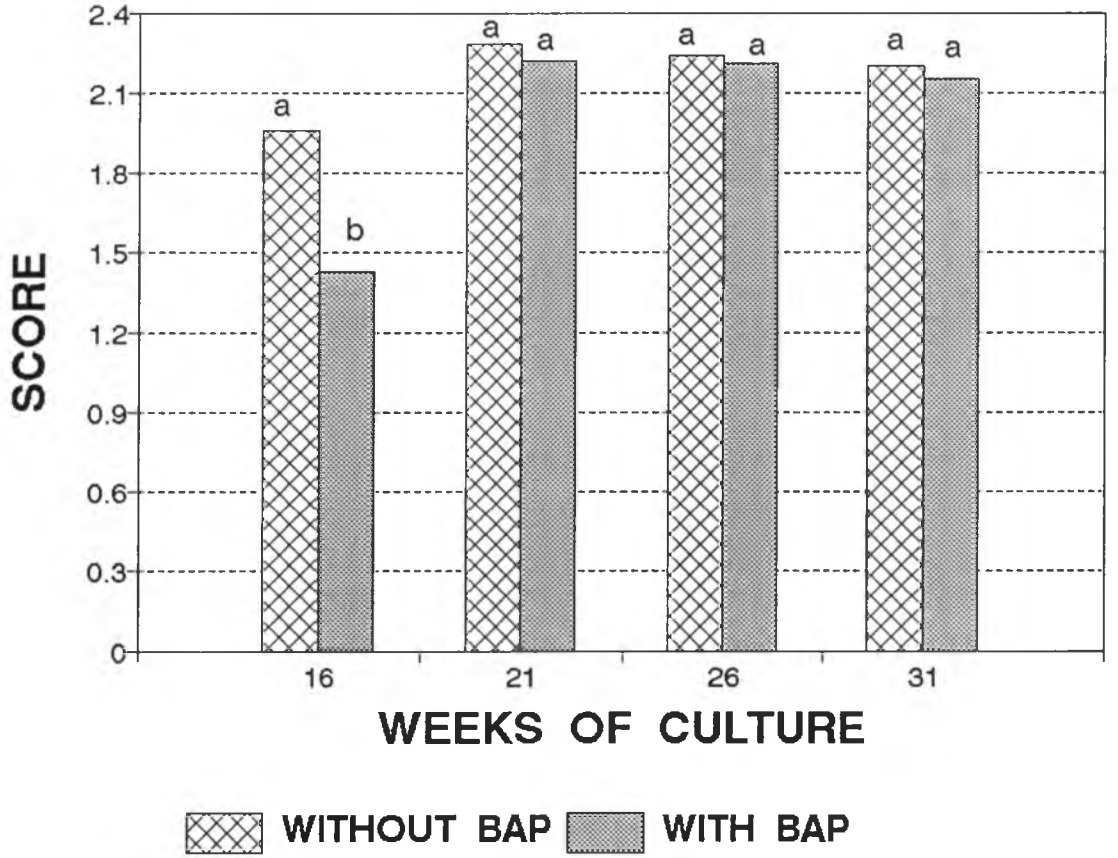


Fig. 14. Effect of 10^{-5}M BAP on tissue browning (same letters are not significant difference at the 5% level).

CHAPTER IV

CALLUS GROWTH AND MORPHOGENESIS

INTRODUCTION

Solid endosperm of coconut starts to form first in the antipodal region, then spreads gradually to the micropylar region. Therefore, the thickness of endosperm prior to maturity is different in the two regions, thicker in the antipodal than in the micropylar tissue. However, thickness is almost the same when the fruit matures. Endosperm in the micropylar region is more highly meristematic than in the antipodal region (Abraham and Mathew, 1963).

High concentration of growth regulators, especially auxins, can induce callus initiation while decreased concentrations may promote differentiation (Reynolds and Murashige, 1979; Tisserat and DeMason, 1980; Sharma et al., 1984; Zaid and Tisserat, 1984; Srinivasan et al., 1985; Rao et al., 1987; Guerra and Handro, 1988). Among auxins, 2,4-D is the most effective, also picloram is similar properties to 2,4-D which induces callogenesis and promotes differentiation in many plants (Beyl and Sharma, 1983; Fitch et al., 1983; Huang and Murashige, 1983; Goodin and Becher, 1987; Valverde et al., 1987).

Although the role of cytokinin has not been defined, it

is commonly added to induce differentiation (Reynolds and Murashige, 1979; Tisserat and DeMason, 1980; Branton and Blake, 1983a; Sharma et al., 1984; Srinivasan et al., 1985; Rao et al., 1987). Among cytokinins, BAP has been applied to promote differentiation in coconut culture (Pannetier and Buffard-Morel, 1982a; Branton and Blake, 1983a; 1986; Sugimura et al., 1988; Sugimura and Salvana, 1989).

Endosperm callus of coconut is considered recalcitrant and only a few cases of morphogenesis have been reported. IAA conjugates (IAA-asp, IAA-ala or their combination) have been applied to induce callogenesis and differentiation in coconut embryo culture (Bhalla-Sarin et al., 1986). Antiauxins (AZI, PCMP, TIBA) as well as ABA have been used to prevent recallusing of embryoids in soapnut (Sapindus trifoliatus) (Desai et al., 1986) and to enhance morphogenesis in pine cultures (Durzan and Gupta, 1987; Boulay et al., 1988; Roberts et al., 1990). Zeatin has been applied to induce morphogenesis on Actinidia chinensis (Harada, 1975; Gui et al., 1988) and combinations of NAA, BAP and GA_3 on Annona squamosa and cassava cultures (Kartha et al., 1974).

MATERIALS AND METHODS

Materials and methods of chapter IV were as reported in

chapter III. For morphogenesis, approximately 1 g of callus from the control was subcultured on basal medium (BM) supplemented with 8.12×10^{-6}M IAA-ala, 6.20×10^{-6}M IAA-asp, combinations of 8.12×10^{-6}M IAA-ala + 6.20×10^{-6}M IAA-asp, 16.93×10^{-6}M AZI, 9.32×10^{-6}M PCMP, 4.00×10^{-6}M TIBA or 7.57×10^{-6}M ABA. Each treatment had 12 replications. Representative cultures of all treatments were subcultured on BM, BM supplemented with 4.56×10^{-6}M zeatin or combination of 5.37×10^{-6}M NAA, 4.44×10^{-6}M BAP and 2.89×10^{-6}M GA_3.

Callus growth was computed by subtraction of the initial tissue fresh weight after from prior to culture divided by original weight. Growth rate was computed from callus growth divided by number of weeks in culture. The data were analyzed with General Linear Model (GLM), Orthogonal Contrast and Contrast procedures of Statistical Analysis System (SAS).

RESULTS AND DISCUSSION

Figure 15 shows that fresh weight of endosperms increased from 0.13 g of original weight to 3.98 g in 9 WOC, 11.29 g in 16 WOC, 21.65 g in 21 WOC, 37.70 g in 26 WOC and 45.67 g in 31 WOC. Growth rate increased from 0.03 $g \cdot week^{-1}$ in 9 WOC to 0.14 g in 16 WOC, 0.27 g in 21 WOC, 0.43 g in 26

WOC and decreased to 0.18 $g \cdot week^{-1}$ in 31 WOC (Table 23 and Figure 16).

Growth rate was significantly different depending on the fruit sources. Figure 17 shows growth rate of tissues from four fruits after 31 WOC. Growth rate of fruits 1 and 2 was significantly higher than the rest. Growth rate between fruit 1 and 2 was not significantly different. Fruits 3 and 4 grew slowly and were nearly equal in their overrall growth (Table 23).

Although the initial weight of micropylar explants (0.10 g) was different from antipodal explants (0.12 g), their growth rates were not significantly different (Figure 18). The growth rate of both types of explants increased substantially from 9th to 26th WOC and dropped off afterwards (Table 23).

When treated with 2,4-D or picloram, growth rate increased substantially from 9th to 26th WOC and then declined (Table 23 and Figure 19).

Table 23 shows the effect of 2,4-D and picloram concentration on growth rate of tissues along duration of cultures. On 9 WOC, growth rate of tissues treated with 10^{-3}M 2,4-D and picloram was significantly less than that of the other concentrations (Figure 20). Growth rate of tissues treated with 10^{-5}M was higher than 10^{-3}M 2,4-D and picloram on 16th WOC but was no significant difference with other concentrations (Figure 21). All concentrations had similar

growth dynamics in that growth rate increased until 25 WOC (Figure 22 and 23). Thereafter, the growth rate of cultures initially exposed to 2,4-D and picloram declined, while growth rate of control continued to increase (Figure 24).

Growth rate of tissues treated with and without 10^{-5}M BAP followed a similar pattern, increased 26th WOC and then declined (Table 23 and Figure 25). The addition of BAP did not cause any significant difference in growth rate.

Attempts to induce morphogenesis by subculture of endosperm callus on BM supplemented with IAA-conjugates, antiauxin, ABA, zeatin or combination of NAA, BAP and GA_3 were unsuccessful.

However, an organized structure developed from endosperm callus derived from antipodal tissue treated with 10^{-6}M picloram after 21 WOC. The explant came from one of the two fruits taken from a tree at the Magoon greenhouse facility. Figure 26 shows the "organ" which was elongate, opaque and contrasted to surrounding yellowish brown callus. The "organ" grew bigger and more elongate after two months (Figure 27). However, growth was very slow. It had several protrusions on its upper surface after 8.5 months (Figure 28).

No color change was observed upon transfer of the "organ" to light. The "organ" became triangular after 12 months (Figure 29). Isolation and transfer of this "organ" to medium with 2.89×10^{-6}M GA_3 + 5.37×10^{-6}M NAA + 4.44×10^{-6}M

BAP did not speed its growth. After 14 months, its diameter was 2 mm to 9 mm long, and the shape changed from triangular to cylindrical. Figure 30 shows this structure after 14 months. The "organ" was removed after 14 months for histological studies.

Figure 31 shows that similar structure also developed from callus derived from the previous experiment with fruits from Moanalua. This callus was initially treated with 207.04×10^{-6}M picloram, subcultured to 8.28×10^{-6}M picloram, then subcultured to 2.26×10^{-4}M 2,4-D and 5×10^{-6}M BAP after 17 months of culture.

Callus growth rate was sigmoid and parallel to tissue browning. This result was also observed in suspension cultures of carrot (Sugano et al., 1975) and birch cultures (Welander, 1988). On the contrary, browning of tissue caused by polyphenol concentration was inversely correlated with growth rate in the tea plant (Forrest, 1969) and grapevine in vitro (Yu and Meredith, 1986). This indicates that plants can have different growth responses to browning.

Growth rate was significantly different among tissues derived from different trees as well as from the same tree, especially in the beginning. Genotype, fruit position in the bunch of a single tree and seasonality may influence explant response. Three months separated harvest of fruits from Magoon and Moanalua. Furthermore, the growth conditions were very different in these locations. Consequently, there are

many uncontrolled factors which could account for these differences.

Growth rate was not significantly different for endosperm from different regions of fruit. Morphogenesis occurred with callus derived from the antipodal region. This did not agree with Abraham and Mathew (1963) who observed that endosperm from the micropylar region was more meristematic than from the antipodal region. However, it is impossible to draw firm conclusions about this since only two organized structures were obtained.

The two types of auxin (2,4-D and picloram) did not cause any significant difference in growth rate. On the contrary, Fitch et al. (1983) reported that growth rate of sugarcane hybrids and Saccharum spontaneum cultures treated with 2,4-D was higher than with picloram. On the other hand, Beyl and Sharma (1983) reported that growth rates of Gasteria and Haworthia treated with picloram was greater than with 2,4-D. This indicates that different plants can respond differently to various auxins.

The addition of BAP also did not cause any significant difference in growth rate. On the contrary, Eeuwens (1978), Kuruvinashetti and Iyer (1980) and Sharma et al (1984) found BAP increased fresh weight of coconut and date palm callus. This could be due to the use a different explants or to other factors outlined above.

Callogenesis occurred in the control which had a higher

growth than other treatments after 31 WOC. Paranjothy and Rohani (1982), Reynolds (1982) and Paranjothy (1986a; 1987) reported that auxin was necessary for induction of callogenesis in coconut. Auxin was clearly not required in this study.

Morphogenesis from endosperm callus occurred after 21 WOC, a relatively short time, compared to Pannetier and Buffard-Morel (1982b) who induced embryoids from leaf cultures of coconut after 6 months of culture. The growth of this organ was very slow. The "organ" formed several lumps on the surface which could have been leaf primordia, shoot apical meristems or somatic embryos.

Morphogenesis in a few cultures out of a large number of cultures was reported in the following cases. Less than one out of 1000 developed roots or plantlets with maize endosperm (LaRue, 1947). One embryo formed out of 452 pear endosperm + nucellus cultures (Janick, 1982), only one callus developed from several thousand coconut anther cultures (Radojevic cited in Kovoor, 1981). One embryo developed from over 200,000 coconut anther cultures (Monfort, 1985; Thanh-Tuyen, 1990), oil palm (Jones, 1974) and date palm (Tisserat, 1979b).

Variation of culture responses from tree to tree and batch to batch, were likely a problem in coconut cultures in this study, was also reported by Blake (1990; 1991), Thanh-Tuyen (1990) and Rao and Ganapathi (1993).

To date, besides the establishment of embryo-derived plant (De Guzman et al., 1983), only one plant derived from culture has survived and been established in the field in the Solomon Islands by Unilever (Smith, 1986; Blake, 1990; Rao and Ganapathi, 1993).

Picloram was thought to be promising for callogenesis and morphogenesis of coconut endosperm. BAP was not necessary to induce callogenesis or organogenesis in coconut endosperm culture.

Long-term culture of coconut callus did not regenerate (Blake, 1990). In preliminary experiments by us, morphogenesis did occur in endosperm calli on medium with picloram after 17 months. Fitch and Moore (1990) maintained the ability to regenerate in hybrid sugarcane culture over 12 months with picloram but not 2,4-D.

Putrescine, even though a small amount (10 $mg \cdot l^{-1}$) was used throughout the culture could have influenced callogenesis and morphogenesis of endosperm cultures. However, we have no way of knowing this because we did not have appropriate controls to measure its effect. Phytagel as a gelling agent possibly may have the potential to facilitate callogenesis as well as morphogenesis. Preliminary studies showed that phytagel was equal or superior to agar.

In all cases, tissue growth increased substantially with culture duration but the growth rate decreased after 31

WOC. This coincided with transfer to growth regulator-free medium and was most likely due to this. Fruit sources greatly influenced growth rate. There were no significant differences in growth rate between micropolar and antipodal tissues, 2,4-D and picloram and with BAP.

The concentration of 2,4-D and picloram slightly influenced growth rate as 10^{-3}M caused significantly less growth after 9 WOC but not after 21 WOC. The control showed significantly faster growth after 31 WOC compared to cultures exposed to either auxin.

Morphogenesis occurred on tissues from different trees treated with low and high levels of picloram after 21 weeks and 17 months of culture. This suggests that picloram has the potential to induce morphogenesis of coconut endosperm callus and may maintain totipotency in long term culture. Putrescine and phytagel possibly facilitated the differentiation.

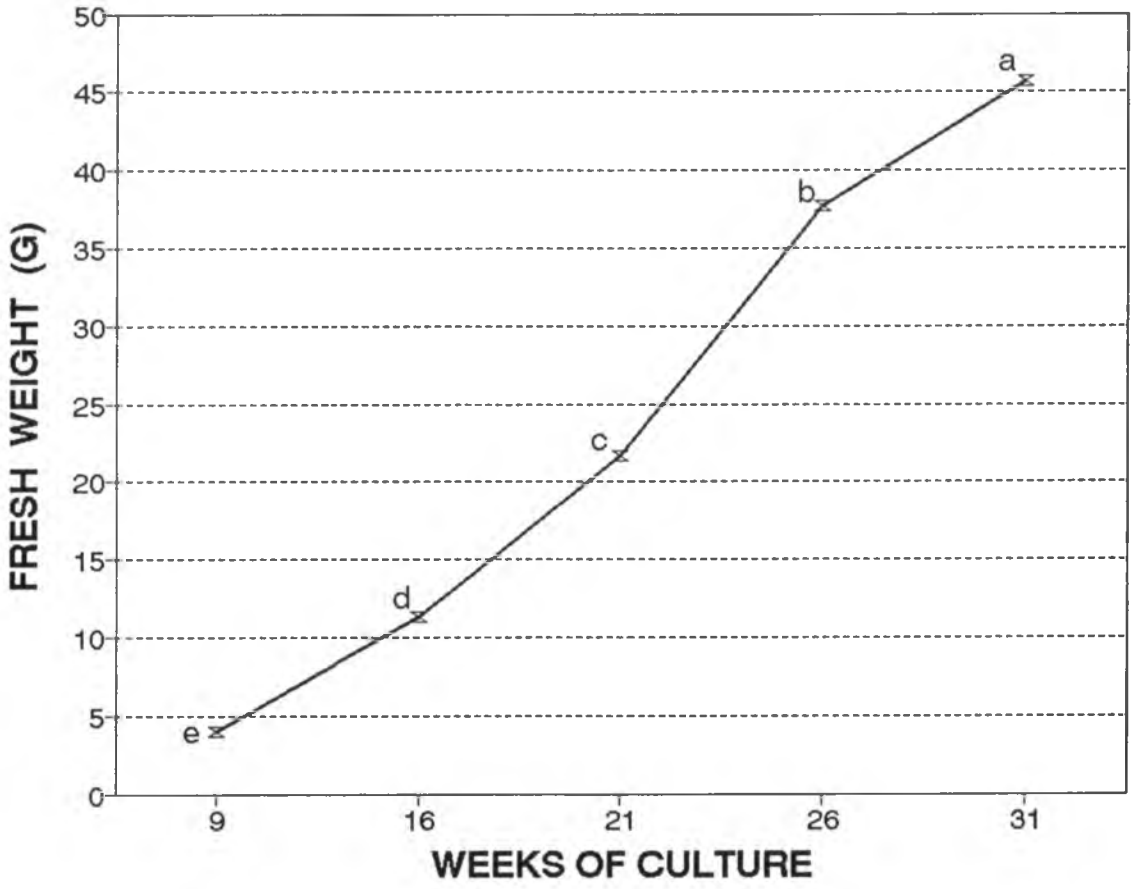


Fig. 15. Average tissue growth of all treatments (comparison between all possible combinations and same letters are not significant difference at the 5% level).

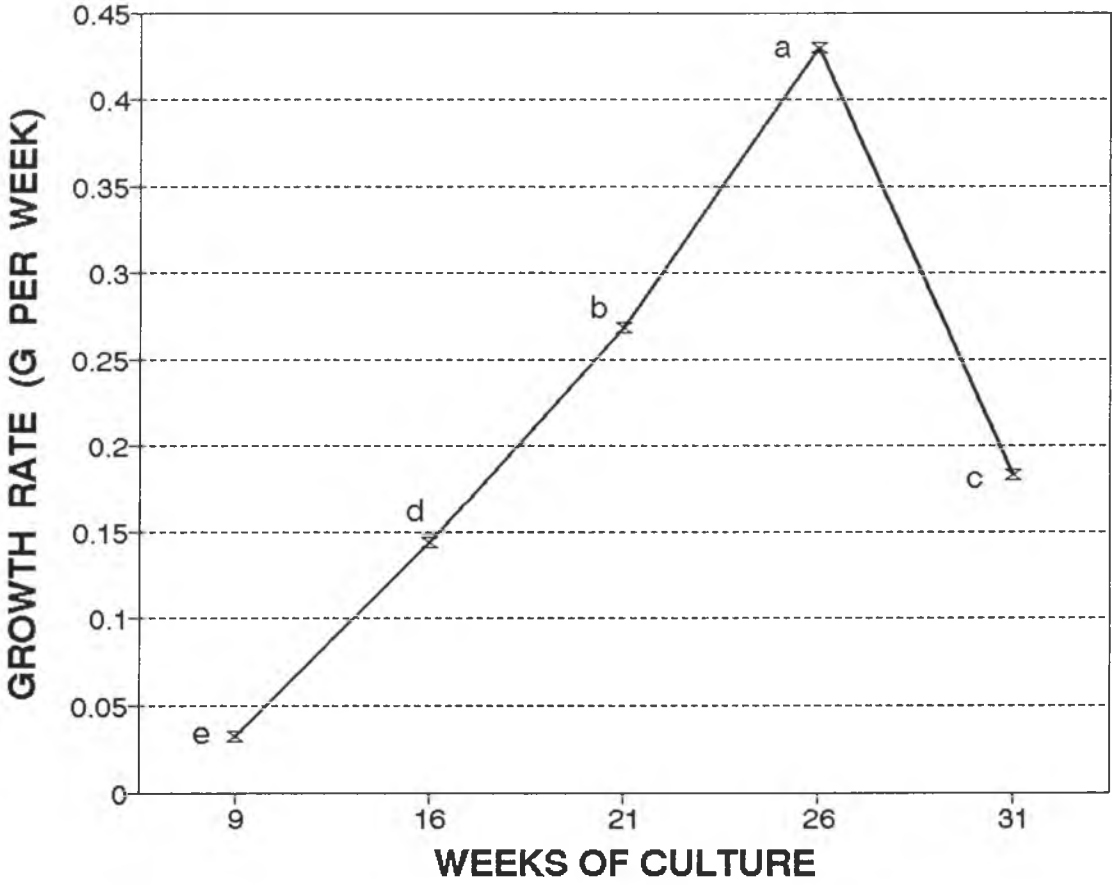


Fig. 16. Average growth rate of all treatments (comparison between all possible combinations and same letters are not significant difference at the 5% level).

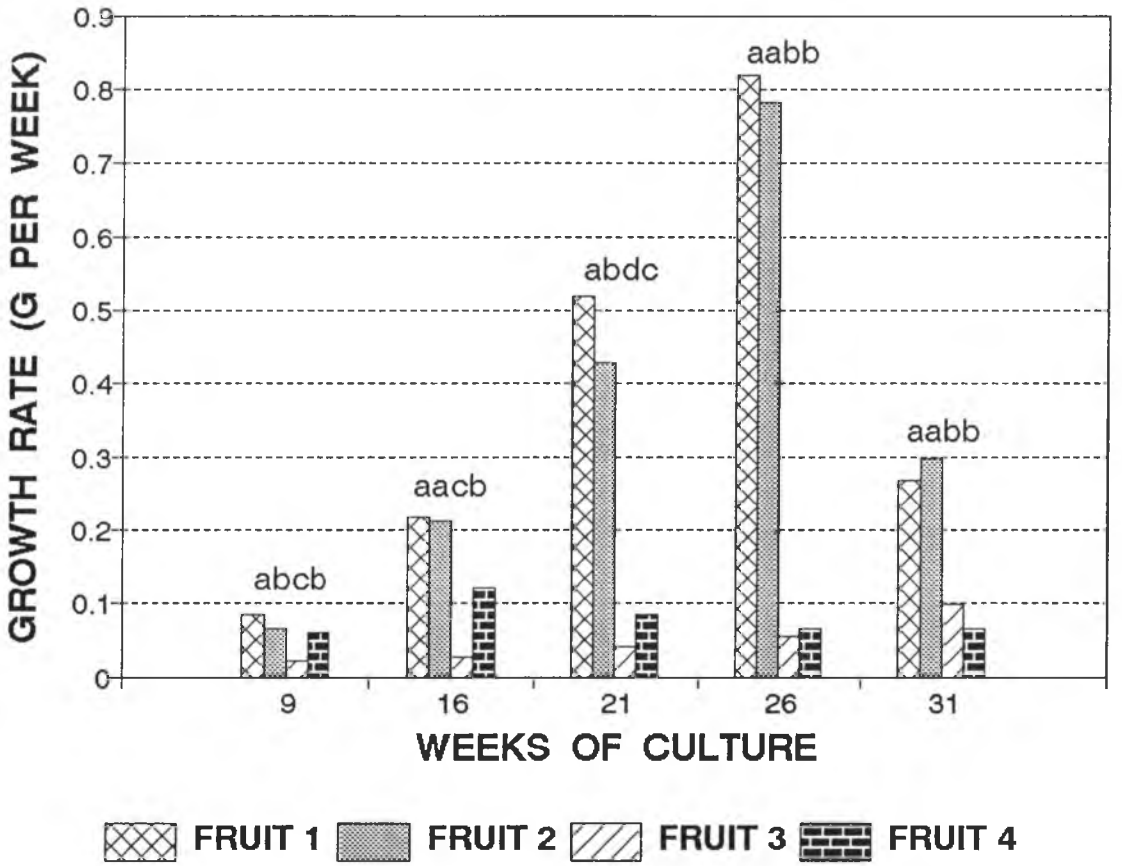


Fig. 17. Growth rate of different fruit sources (same letters are not significant difference at the 5% level).

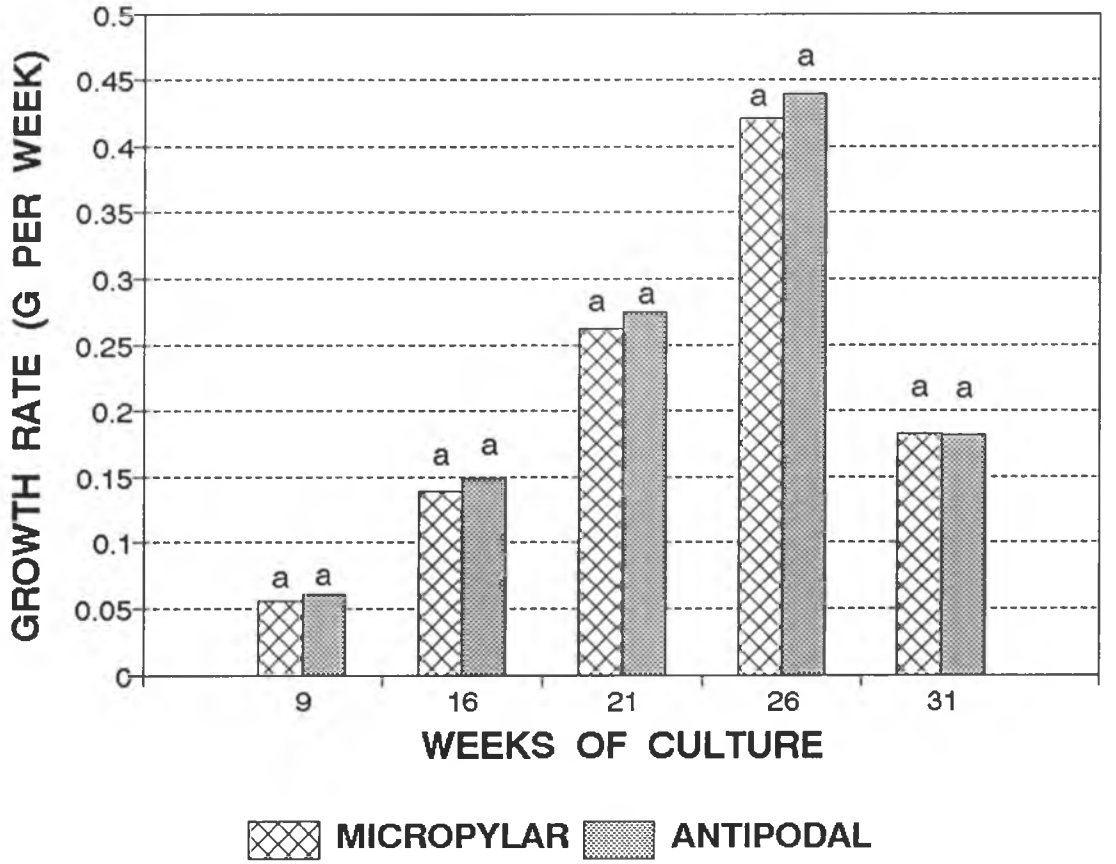


Fig. 18. Effect of endosperm region on growth rate (same letters are not significant difference at the 5% level).

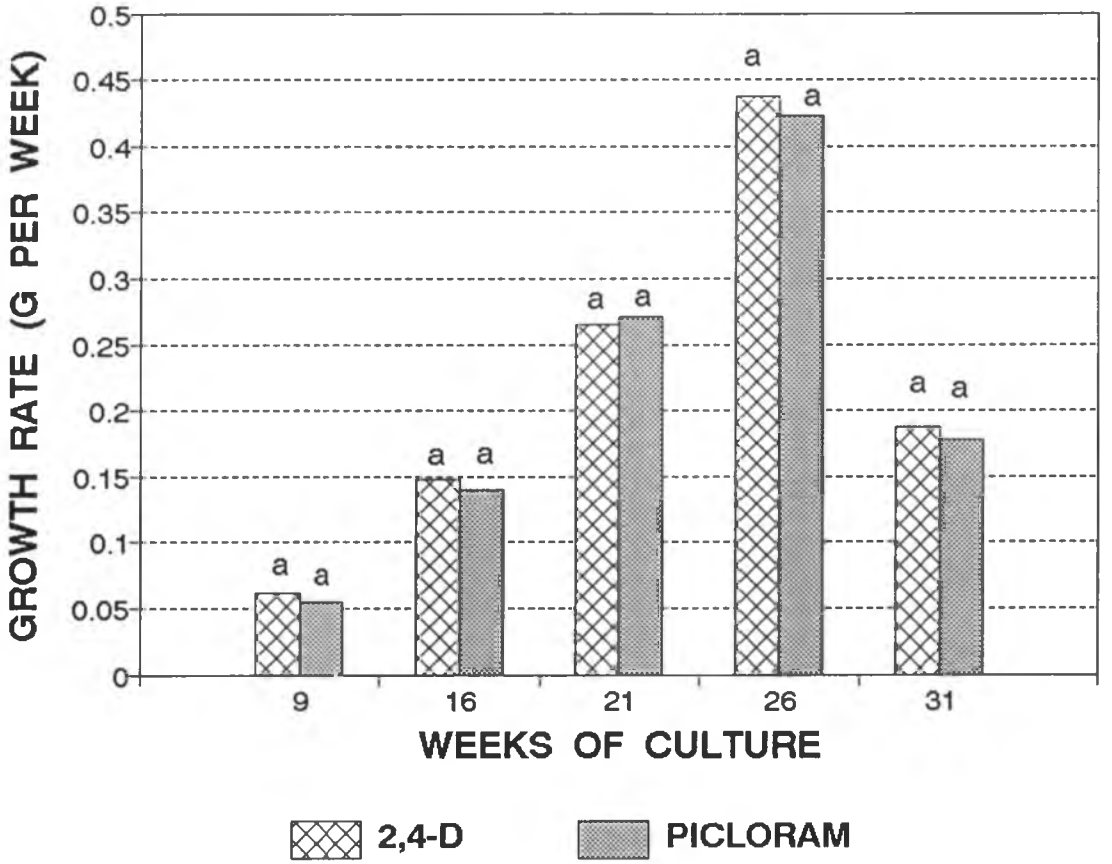


Fig. 19. Effect of 2,4-D and picloram on growth rate (same letters are not significant difference at the 5% level).

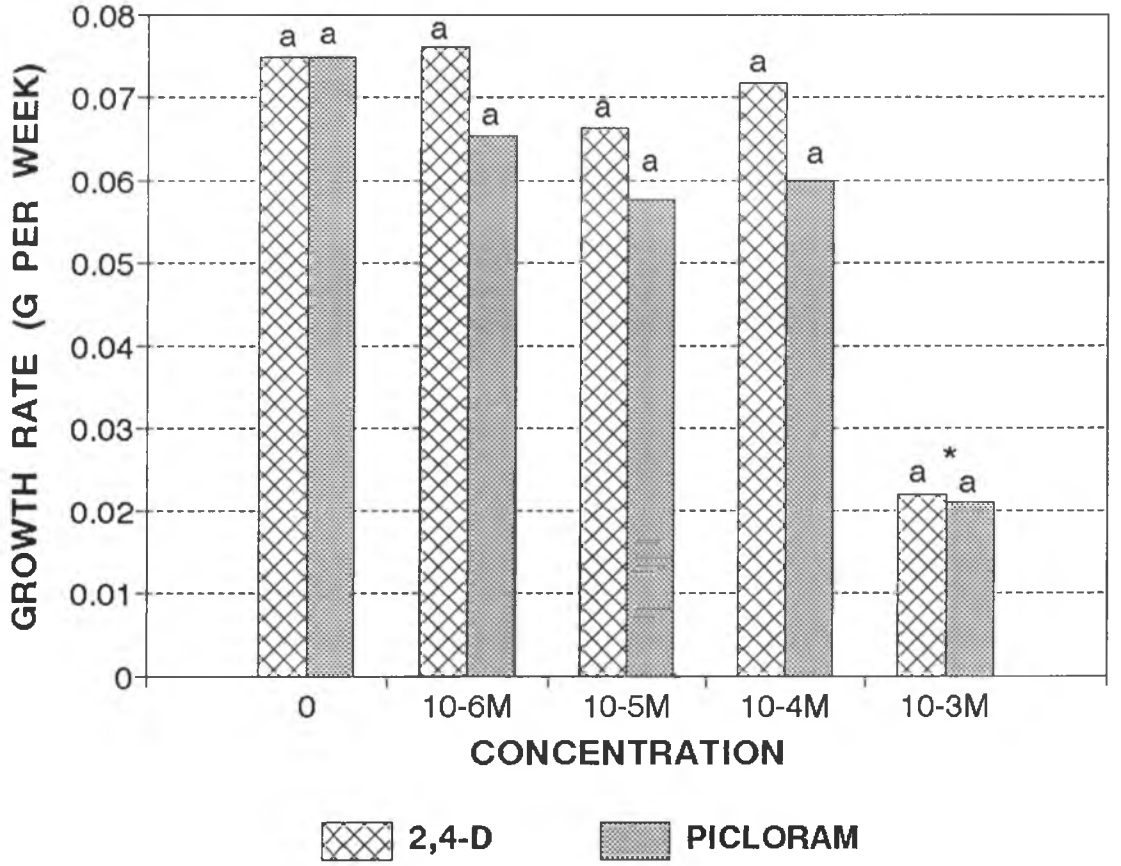


Fig. 20. Effect of 2,4-D and picloram concentrations on growth rate after 9 weeks of culture (same letters are not significant difference within and * is significant difference among auxin concentrations at the 5% level).

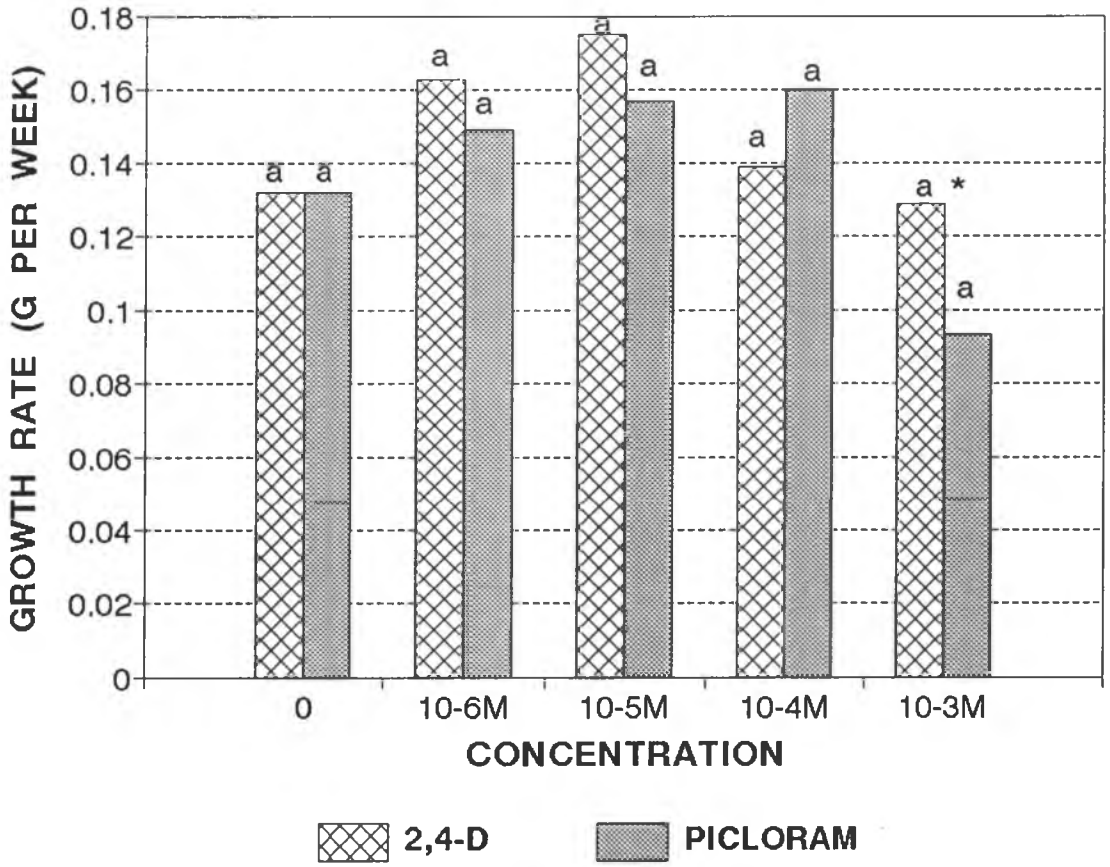


Fig. 21. Effect of 2,4-D and picloram concentrations on growth rate after 16 weeks of culture (same letters are not significant difference within and * is significant difference among auxin concentrations at the 5% level).

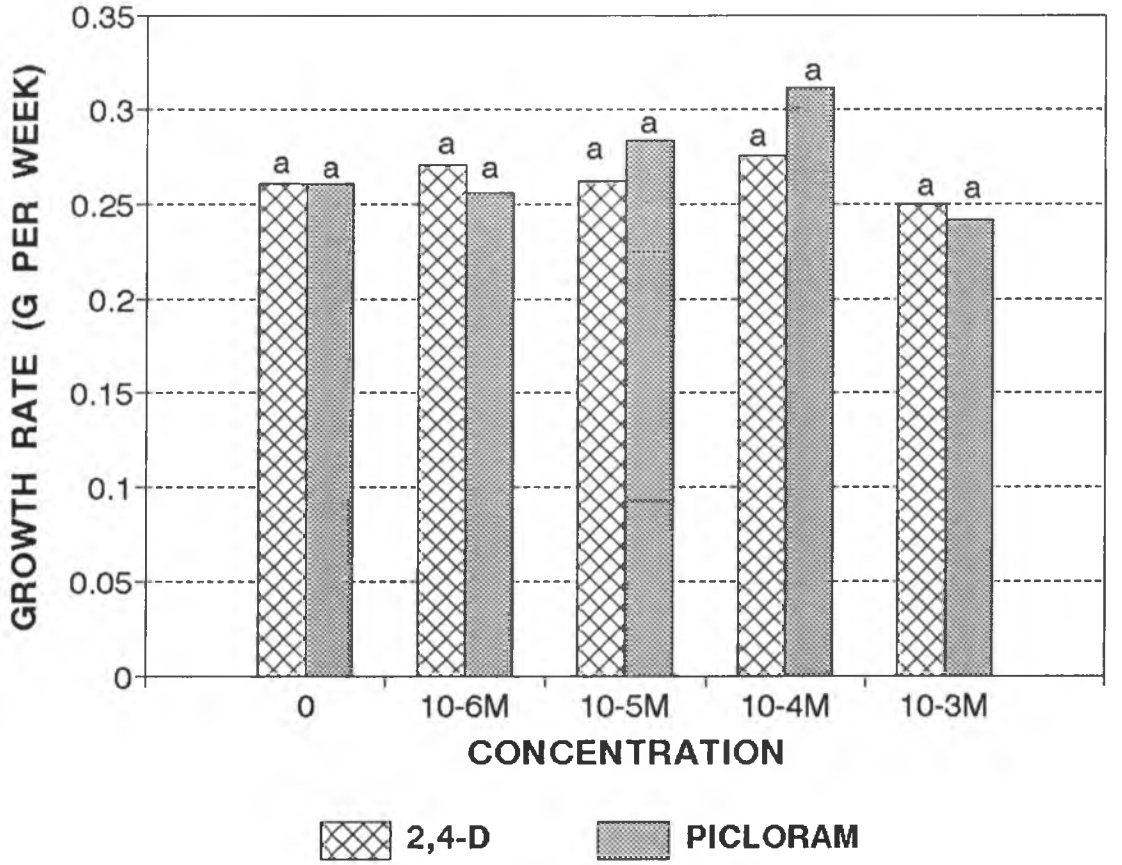


Fig. 22. Effect of 2,4-D and picloram concentrations on growth rate after 21 weeks of culture (same letters are not significant difference at the 5% level).

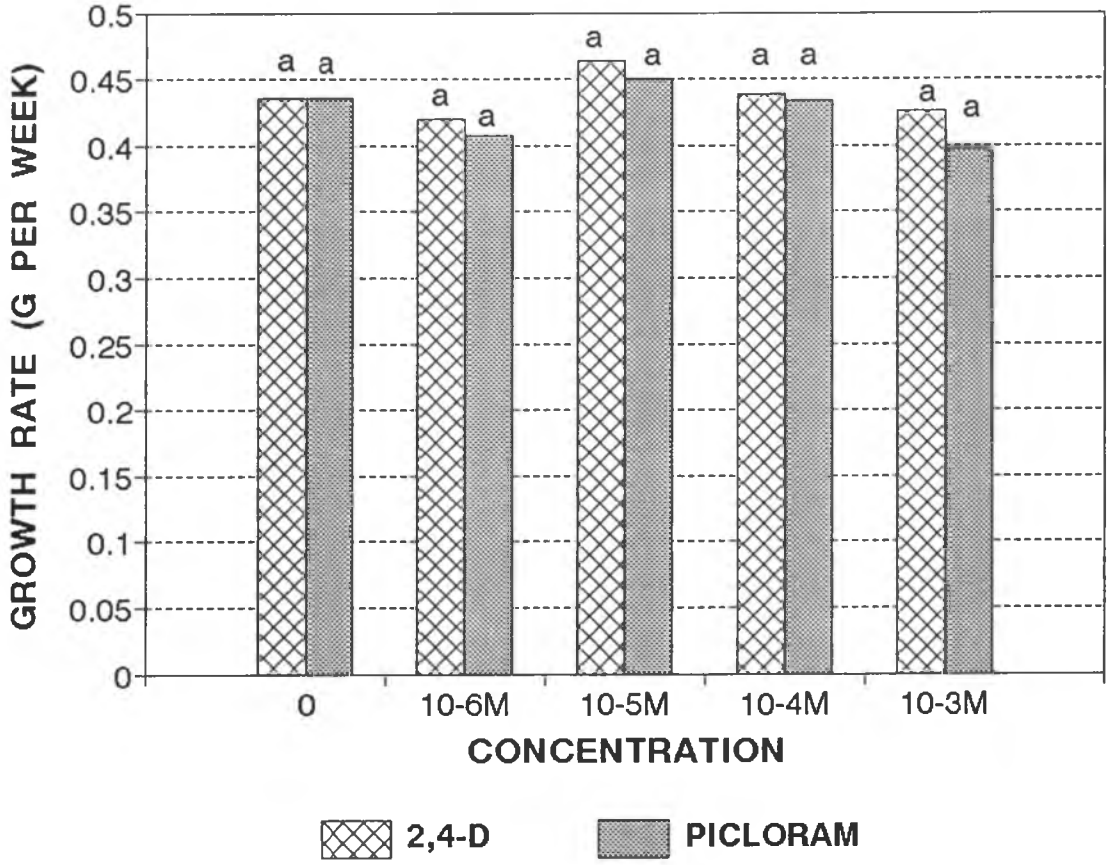


Fig. 23. Effect of 2,4-D and picloram concentrations on growth rate after 26 weeks of culture (same letters are not significant difference at the 5% level).

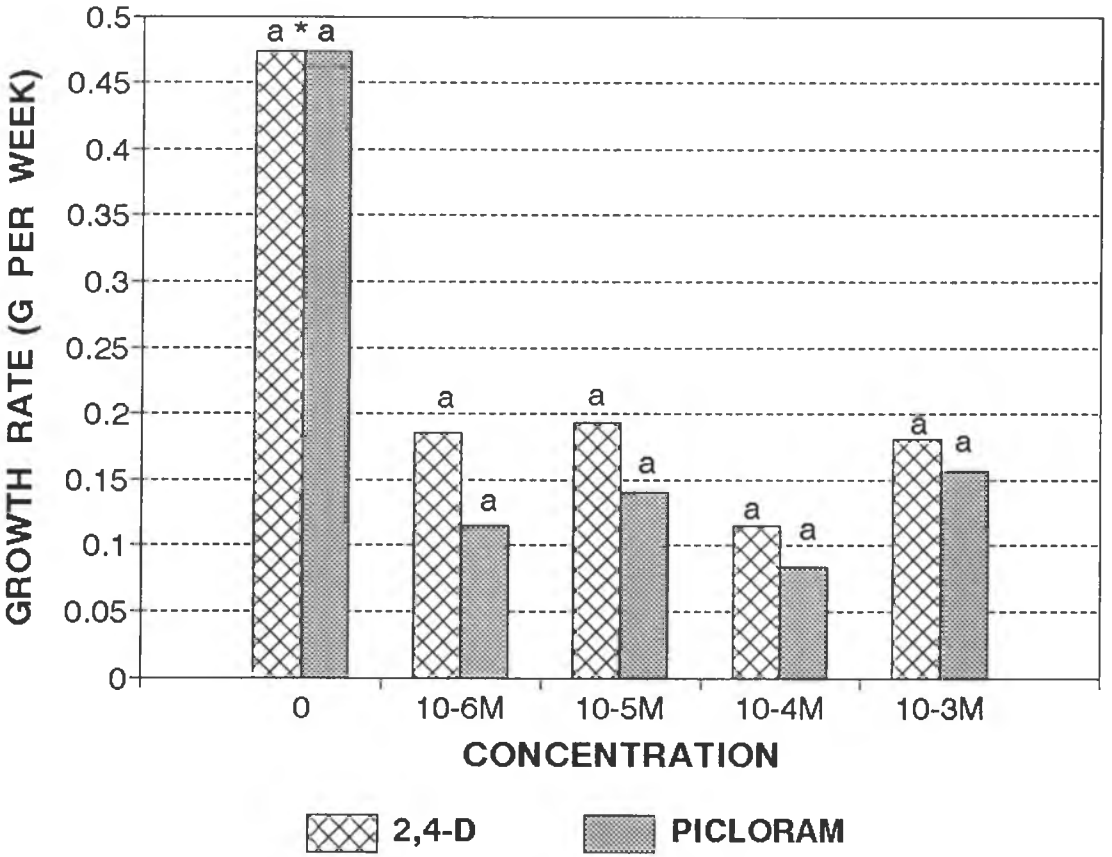


Fig. 24. Effect of 2,4-D and picloram concentrations on growth rate after 31 weeks of culture (same letters are not significant difference within and * is significant difference among auxin concentrations at the 5% level).

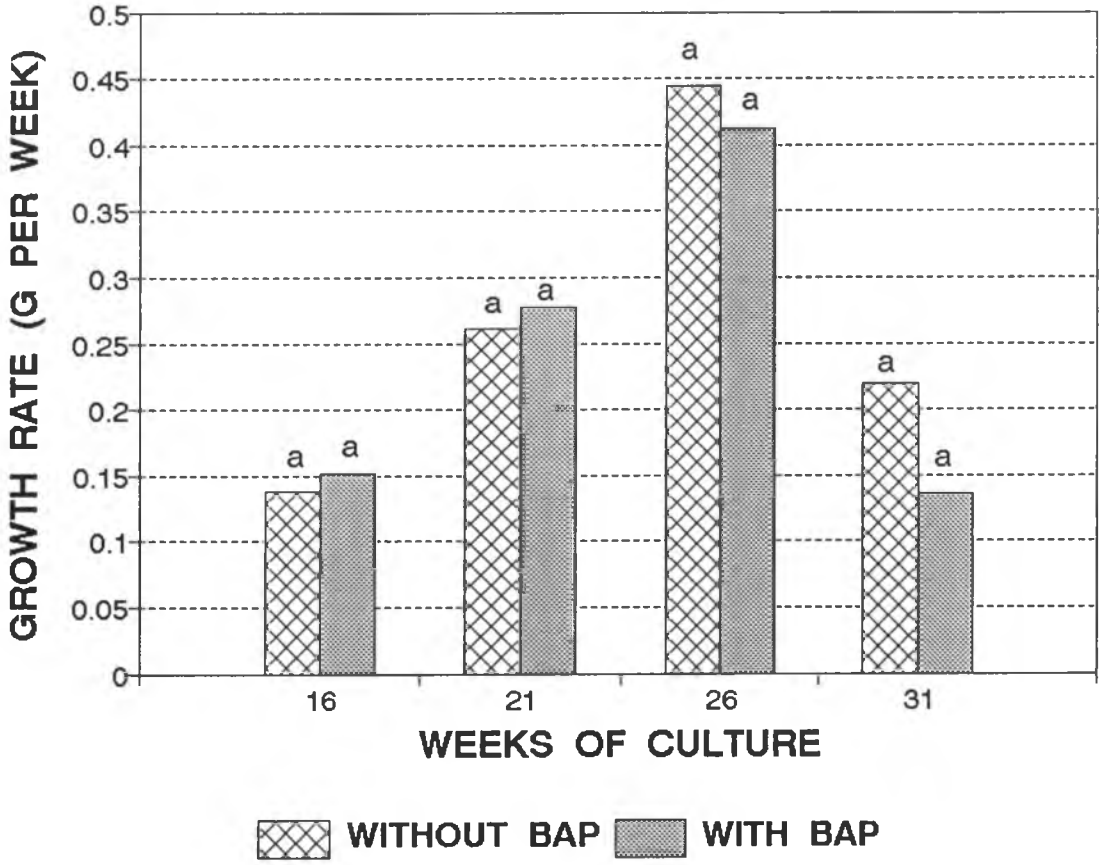


Fig. 25. Effect of 10^{-5}M BAP on growth rate (same letters are not significant difference at the 5% level).

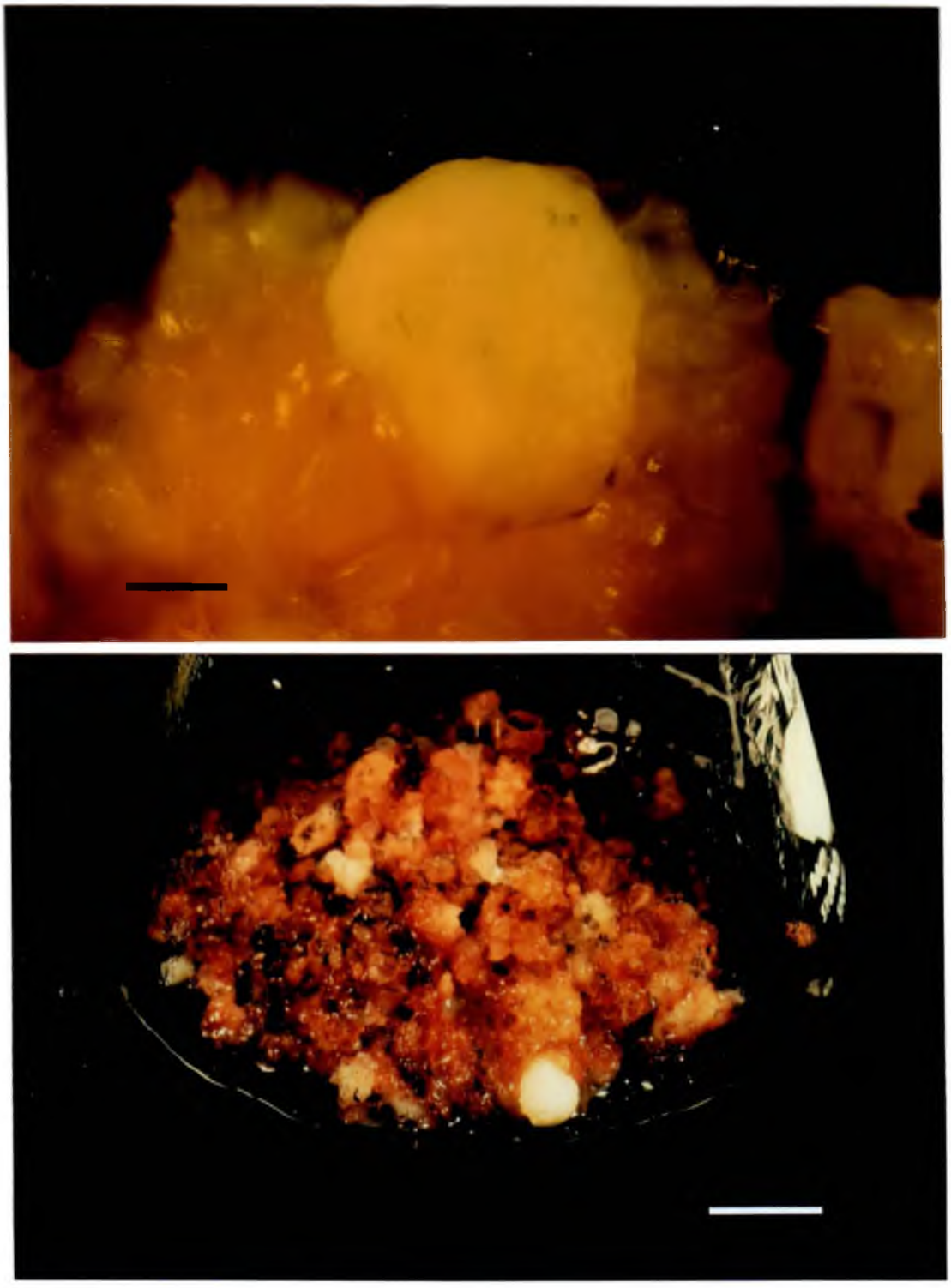


Fig. 26. The first appearance of a morphogenesis from antipodal tissue treated with 10^{-6}M picloram after 21 weeks of culture. Bar represents 1 mm.

Fig. 27. The "organ" shape became more elongate after 2 months. Bar represents 1 cm.

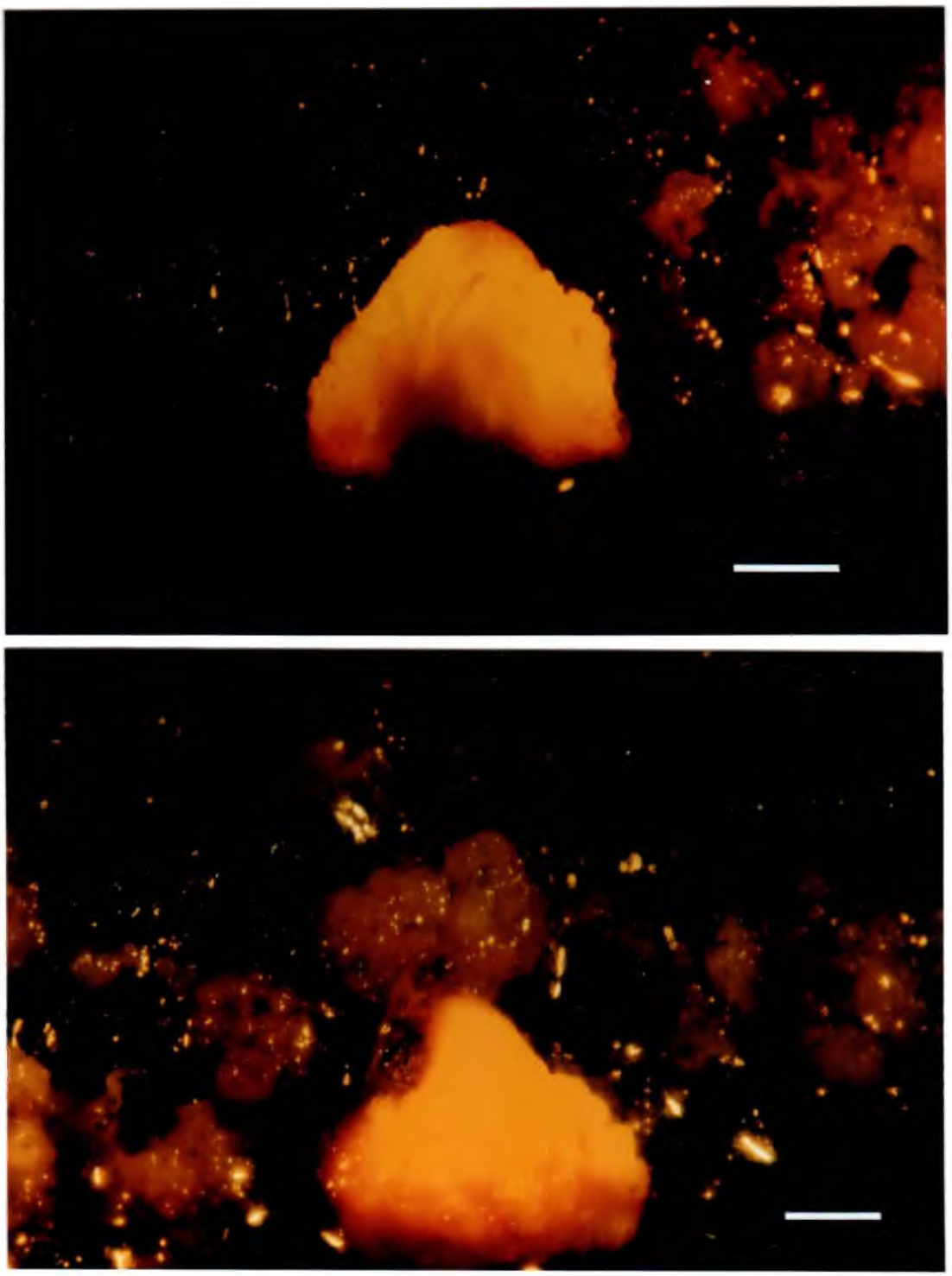


Fig. 28. The "organ" with several lumps on the surface after 8.5 months. Bar represents 3 mm.

Fig. 29. The "organ" became a triangular shape after 12 months. Bar represents 3 mm.

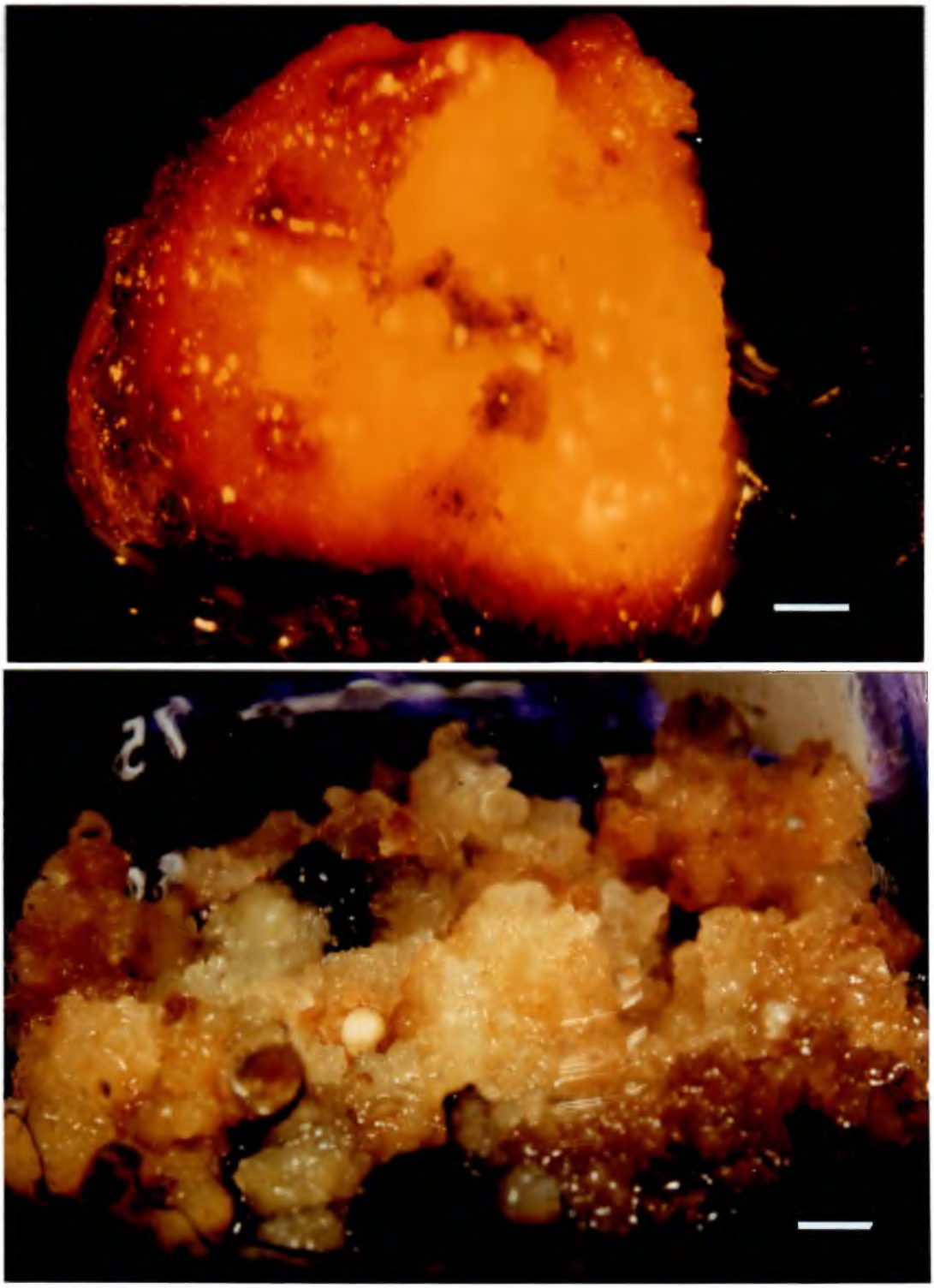


Fig. 30. The "organ" became elongate shape after 14 months. Bar represents 1 mm.

Fig. 31. Another morphogenesis occurred from tissue treated with 207.04×10^{-6}M picloram after 17 months. Bar represents 3 mm.

CHAPTER V

HISTOLOGICAL STUDY

INTRODUCTION

Before differentiation is visually recognizable, many changes occur at the cellular and tissue levels. Cells can undergo a series of orderly divisions, to form "promeristemoids" (Villalobos et al., 1985; Flinn et al., 1988) or meristemoids (Ross et al., 1973; Murashige, 1974). The latter aggregation consists of small, isodiametric, thin-walled, micro-vacuolated cells, highly basophilic, densely staining nuclei, nucleoli and cytoplasm (Thorpe and Murashige, 1970; Reinert et al., 1977;). They may also contain numerous starch grains or lipid deposits (Thorpe and Murashige, 1970; Ross et al., 1973; Villalobos et al., 1985; Arnold and Hakman, 1988). Meristemoids may develop as embryos, organs or vascular tissues (Reinert et al., 1977; Thorpe, 1978).

There are two basic types of cells in callus. These may be classified as morphogenic and nonmorphogenic. Morphogenic cells may produce larger organized entities which develop into shoots, roots or embryos. Nonmorphogenic cells may also form large masses but these never produce organs or embryos. Morphogenic cells have cytological features similar to those

described above for meristemoid cells. Nonmorphogenic cells are large and diverse shapes, tend to have large central vacuoles, peripheral cytoplasm, faintly visible nuclei and contain mature chloroplasts with grana. Callus composed by embryogenic cells (EC) are compact, white-pale in color and nodular surface, glossy and mucilaginous; whereas callus composed of nonembryogenic cells (NEC) are granular, friable, smooth, soft, wet, translucent, brownish or green (Murashige, 1974; Smith and Street, 1974; Reinert et al., 1977; Durzan and Gupta, 1987; Becwar et al., 1988; Vasil, 1988; Pareddy and Petolino, 1990; Webb and Flinn, 1991).

There are, however, some exceptions, such as the translucent cells in embryogenic conifer calli (Durzan and Gupta, 1987; Becwar et al., 1988), pale green embryogenic callus in parsley (Masuda et al., 1977) and friable embryogenic callus in Phalaenopsis (Kim, 1994); whereas compact and hard was NEC in Douglas-fir cultures (Durzan and Gupta, 1987).

Since few large organized structures were obtained, microscopic observations were taken to determine what types of cell growth and organized development occurred in endosperm callus.

MATERIALS AND METHODS

Solid endosperm of fresh young coconut (7 months postanthesis) and some calli of the 3 months old were cut with a sharp and clean razor blade inside a petri plate containing a small volume of glutaraldehyde fixative. Thin tissue sections (1 - 2 mm) were placed under vacuum two times for 1 hour each. Tissues were washed three times with buffer for 10 minutes each and stored in the refrigerator overnight. Then the tissues were fixed with 2% osmium tetroxide and washed three times with buffer. Specimens were dehydrated in a graded series of ethanol (10% to 70%) for 15 minutes each. Specimens were stored overnight in 70% ethanol in the refrigerator in stoppered vials.

The specimes were dehydraded with graded series of ethanol (70%, 80%, 90%, 95%, and 100%) in 30 minute steps and transferred into vials containing 5 ml of 100% ethanol and 1 ml historesin (HR) and mixed by swirling, capped in vials and stored overnight at room temperature. HR was added daily in increasing volume (3 ml, 3 ml, 4 ml), finally in 2 changes of 5 ml of 100% HR.

The embryo-like "organ-like" structure discussed above derived from endosperm callus, measured 9 mm in diameter and 10 mm in height. It was cut longitudinally into three parts and fixed immediately in a 50 ml mixture of 1% acrolene, 2% glutaraldehyde, 2% paraformaldehyde and 0.05M sodium

cacodylate buffer at pH 7.6 and placed twice under vacuum for 2 days. The fixative was decanted, the tissue was washed three times with buffer (0.05M cacodylate) for 30 minutes each, and stored in the refrigerator for several days. The specimens were completely dehydraded and embedded as described above.

The specimens were transferred to peel-away molds, aluminum pans or beem capsules containing embedding medium. The containers were closed to exclude oxygen and placed in vacuum overnight. Polymerized blocks were glued on wood blocks or plastic rods. These were sectioned with a rotary microtome at 5 - 10 μ. The sections were stained with toluidine blue, a combination of toluidine blue and acid fuchsin, Feulgen-fast green or periodic acid Schiff (PAS). Polymount was added and a cover slip was applied. Water was used as a mounting fluid in some cases. In these cases, the edge of the cover slip was sealed with nail polish.

Stains were applied directly to samples taken from seven month-old liquid cultures. Cell suspensions were pippeted onto a glass slide. A few drops of potassium iodide-iodine (IKI) was added and covered with a coverglass. The specimens were examined and photographed with a Zeiss photomicroscope.

RESULTS AND DISCUSSION

Figure 32 and 33 show that endosperm cells from young coconut fruit are relatively uniform in shape and size. Nuclei have up to five nucleoli. In culture, the cells vary in shape and size (Figure 34).

Figure 34 shows differential staining of endosperm callus cells by acid fuchsin and toluidine blue. The tissue on the right side of Figure 34 are darker than those to the left. Most of the darkening is associated with the cell walls and peripheral cytoplasm (Figure 34). Acid fuchsin stains most cell components, especially mitochondria. Toluidine blue was used as a counter stain (Gurr, 1965). The dark areas of cytoplasm have an accumulation of lipid droplets (Figure 35), while cells in the light area of callus have fewer lipid droplets and have more frequent cell divisions (Figure 36).

Division occurred in many planes (Figure 36 and 41). Some nucleoli appeared long (Figure 36). Figure 37 shows various round cell clusters which have from three to five cells each. Figure 38 shows a linear file of approximately four cells. A structure in Figure 39 resembles a young embryo with suspensor.

Figure 40 show the formation of structures which

resemble promeristemoids (Villalobos et al., 1985; Flinn et al., 1988). Promeristemoid was a term proposed by Villalobos et al. (1985) to describe the formation of the organized cluster of six to eight cells in the early stage *in vitro* of shoot formation by *Pinus radiata* cultures. Similar structures were observed by Flinn et al., (1988) with *P. strobus*.

Larger structures called meristemoids were also observed. Figure 41 shows endosperm callus formed a meristemoid that consisted of a nine celled cluster which heavily stain with PAS due to containing carbohydrates (Gurr, 1965) and over nine celled cluster (Figure 42 and 43).

Longitudinal section of the "organ-like" structure indicated the presence of central vascular system, a parenchymatous cortex and a dermal layer (figure 44 and 46). Figure 45 shows stem tip between sheathing base of cotyledon and vascular tissue toward stem tip. Figure 46 shows vascular tissue containing tracheids. Formation of vascular tissue in the "organ-like" structure of endosperm callus is an indication of organogenesis or embryogenesis. Indeed the "organ-like" structure resembled a zygotic coconut embryo at the advanced stage with sheathing base completely enclosing the stem tip (Haccius and Philip, 1979).

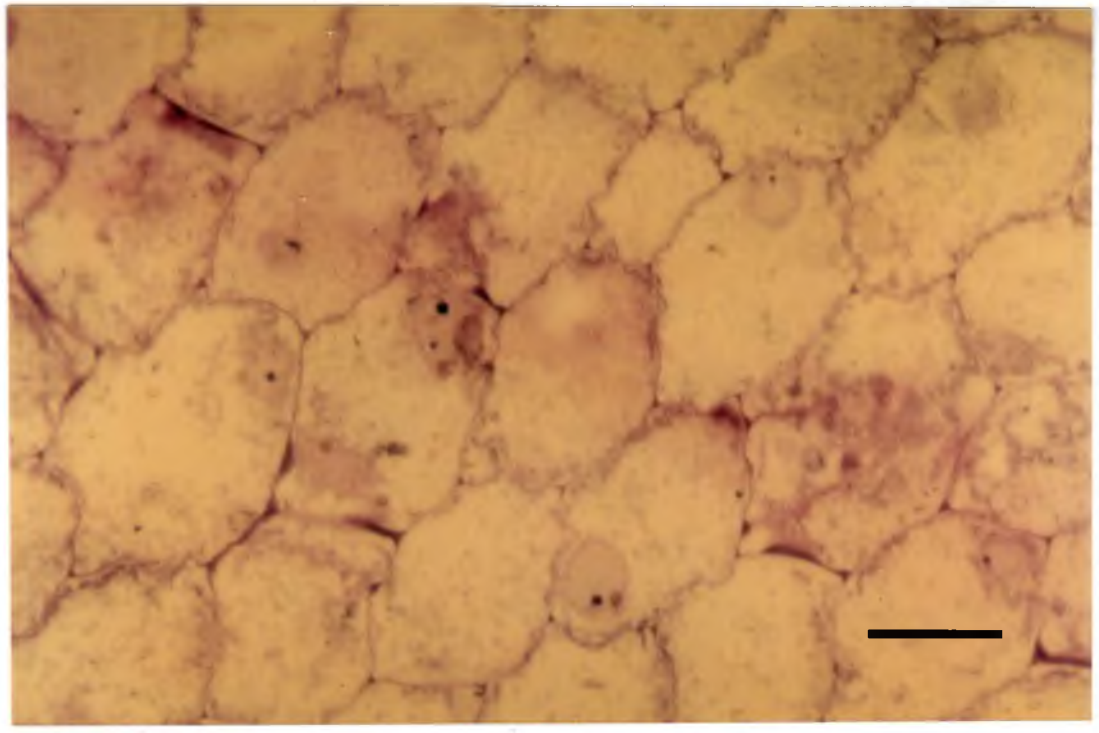
Closer examination of the dermal layer showed that in some areas it contained a meristematic mantle of closely

packed cells subtended by a zone of loosely packed cells (Figures 47 to 54). Many protuberances were visible in the mantle. Some of these resembled early stages of embryo differentiation (Figure 53), while others looked like primordial shoot apices (Figure 54).

The peripheral cells, especially the organized protuberances differed from cells in the adjacent internal region. The mantle cells were smaller, more densely cytoplasmic and had prominent dark nuclei. Similar cells of leaf and hypocotyl cultures in Torenia fournieri and Anagallis arvensis were destined to become embryos, buds and shoot apices (Reinert et al., 1977). Figure 54 shows a protuberance which appears to have a one layered tunica which covers a large group of irregularly-arranged cells which could be the corpus. This organization is only found in shoot apical meristems and leaf primordia. It is impossible to distinguish between these two possible structures in this case. However, the tunica corpus of this "organ-like" was similar to the shoot apex of Phoenix canariensis and P. dactylifera (Ball, 1941). It is possible that early stages of shoot organogenesis occurred in coconut endosperm callus.

The shape of the suspensors on putative somatic embryos was slightly different from those of coconut embryos in vivo but the section shape of the "organ" was similar to the advanced stage of coconut embryo (Haccius and Philip, 1979).

A structure of seven or more celled clusters on coconut endosperm callus (Figure 40 to 43) was similar to promeristemoids which lead to shoot formation with Pinus taeda (Gupta and Durzan, 1987), P. radiata (Villalobos et al., 1985) and P. strobus cultures (Becwar et al., 1988; Flinn et al., 1988; Webb and Flinn, 1991).



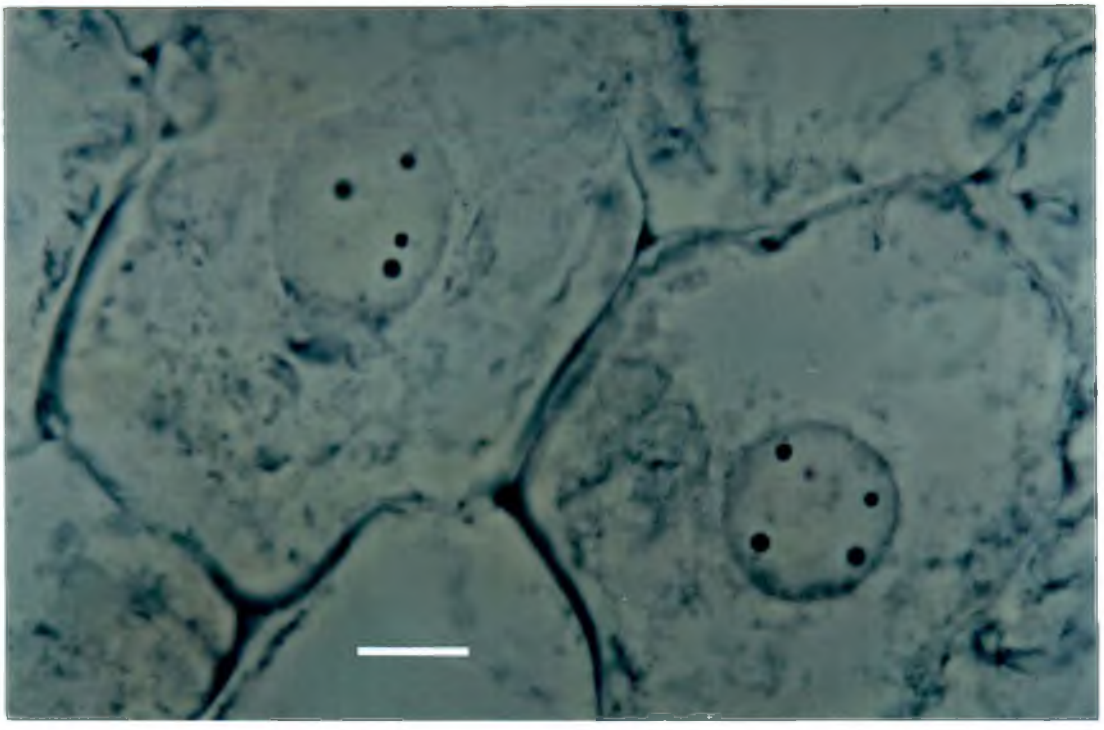


Fig. 32. Endosperm cells of young coconut shows relatively uniform in shape and size fruit, stained with toluidine blue. Bar represents 4 μ.

Fig. 33. Nuclei of endosperm cells consists of 4 - 5 nucleoli, unstained. Bar represents 10 μ.

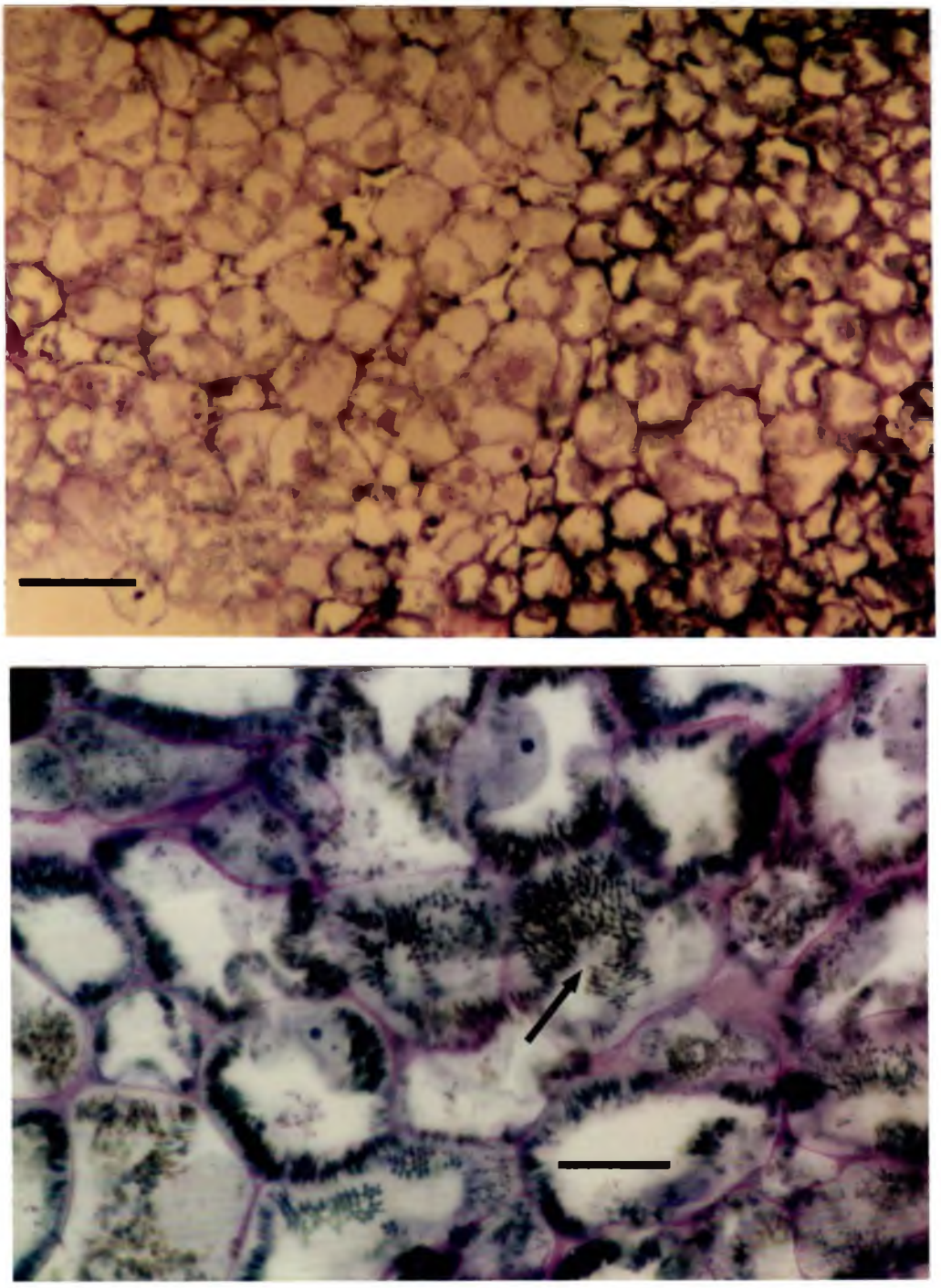
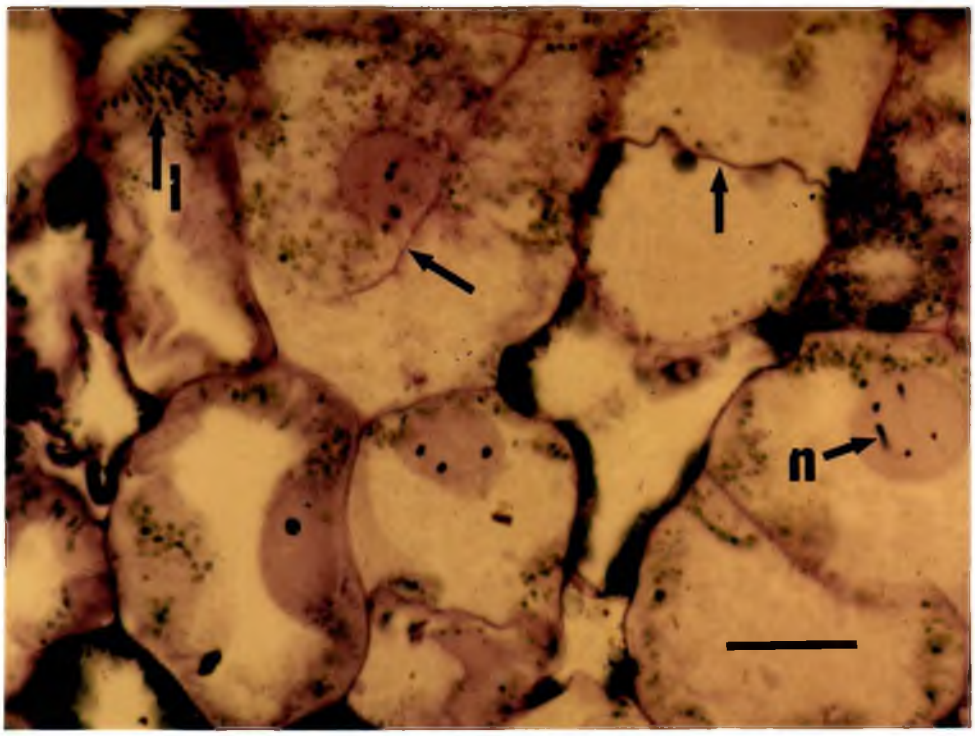


Fig. 34. Cells of endosperm callus shows dark and light areas with cells vary in shape and size, stained with acid fuchsin and toluidine blue. Bar represents 10 μ.

Fig. 35. Enlargement of dark area of Fig. 34. Cells with a lot of lipid droplets. Bar represents 2 μ.



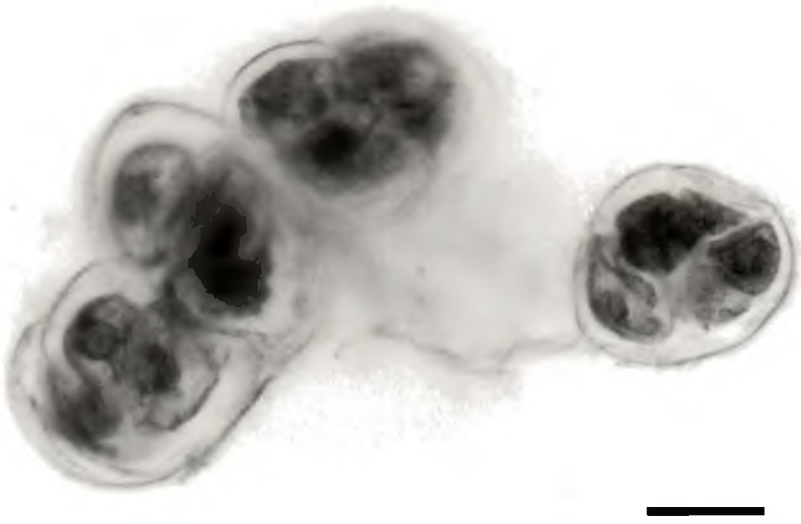
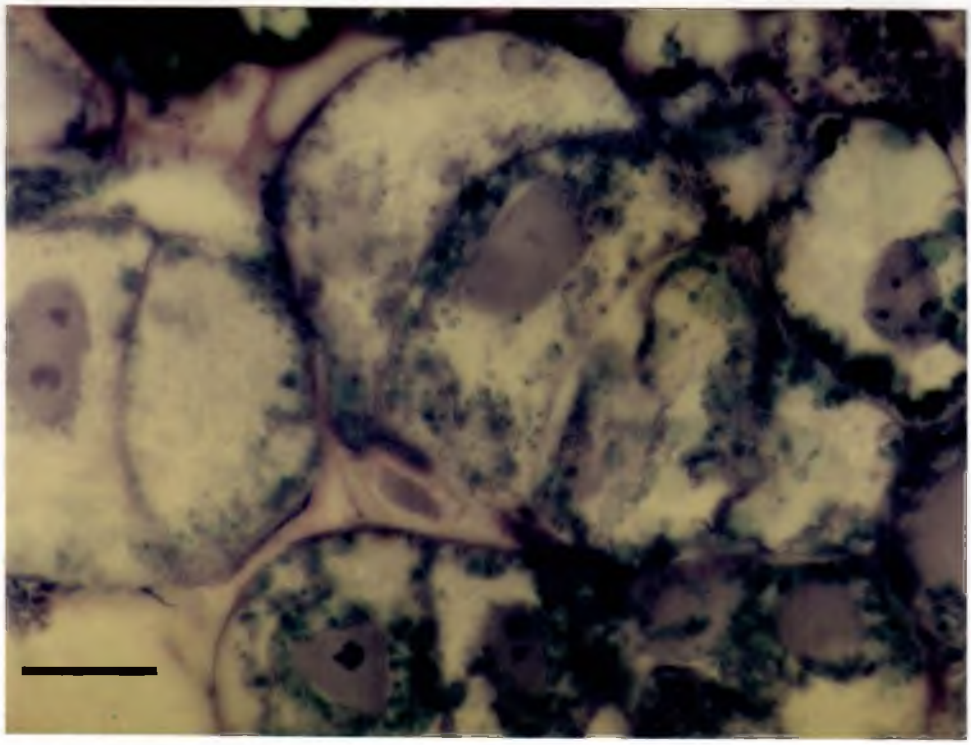


Fig. 36. Enlargement of light area of Fig. 34. Cells underwent many divisions with few lipid droplets (l) and long nucleoli (n). Bar represents 2 μ.

Fig. 37. Various round cell clusters with 3 - 5 cells of coconut endosperm treated with 10^{-5}M 2,4-D, stained with IKI. Bar represents 4 μ.



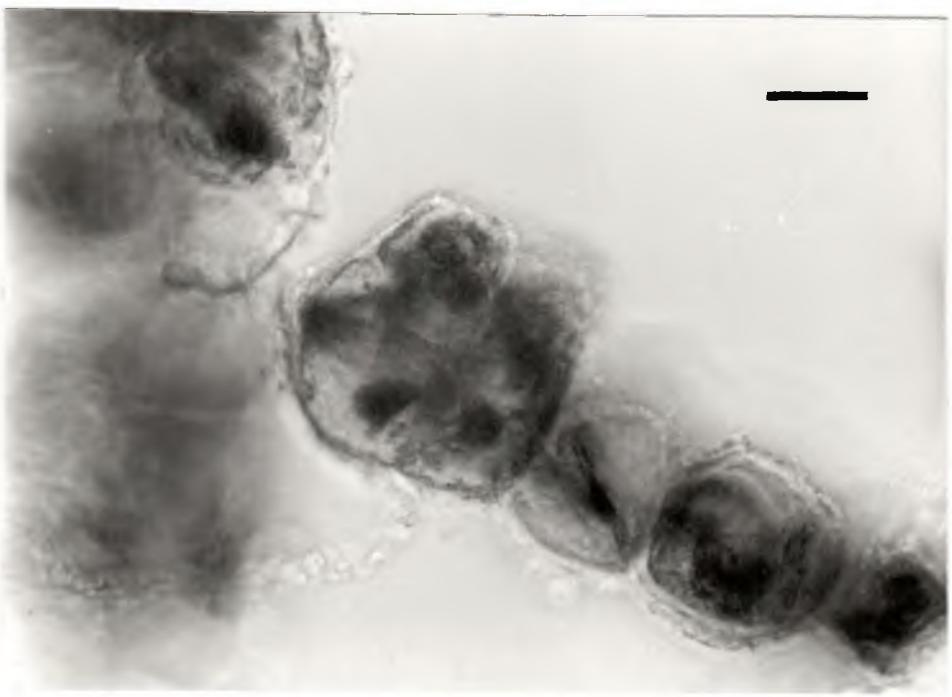
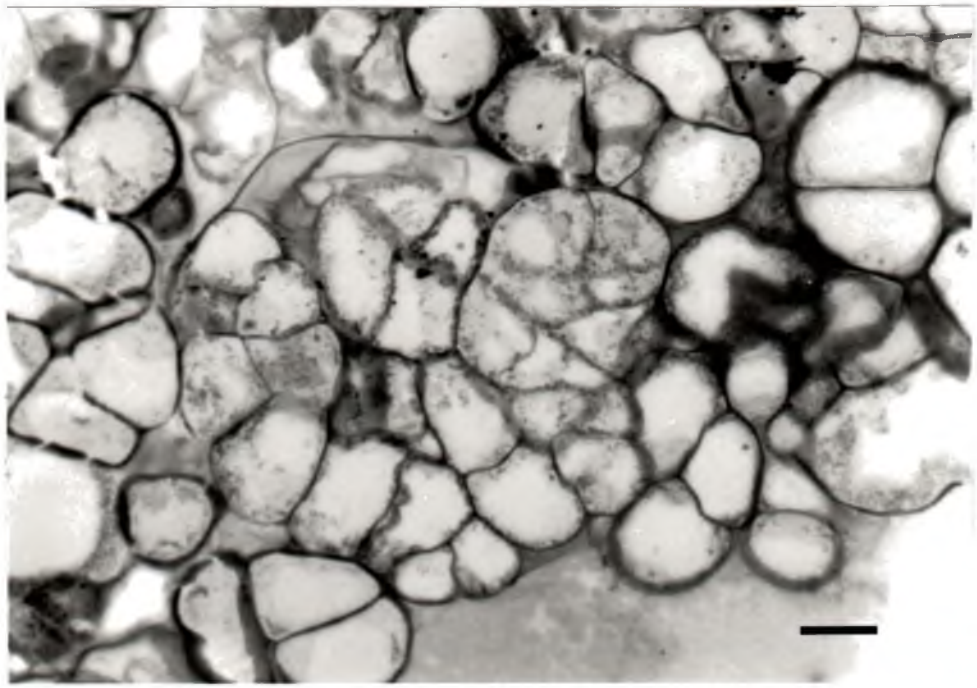


Fig. 38. Formation of linear four cells structure, stained with acid fuchsin and toluidine blue. Bar represents 20 μ.

Fig. 39. Structure resembles a young embryo with suspensor of coconut endosperm treated with 10^{-5}M 2,4-D, stained with IKI. Bar represents 2 μ.



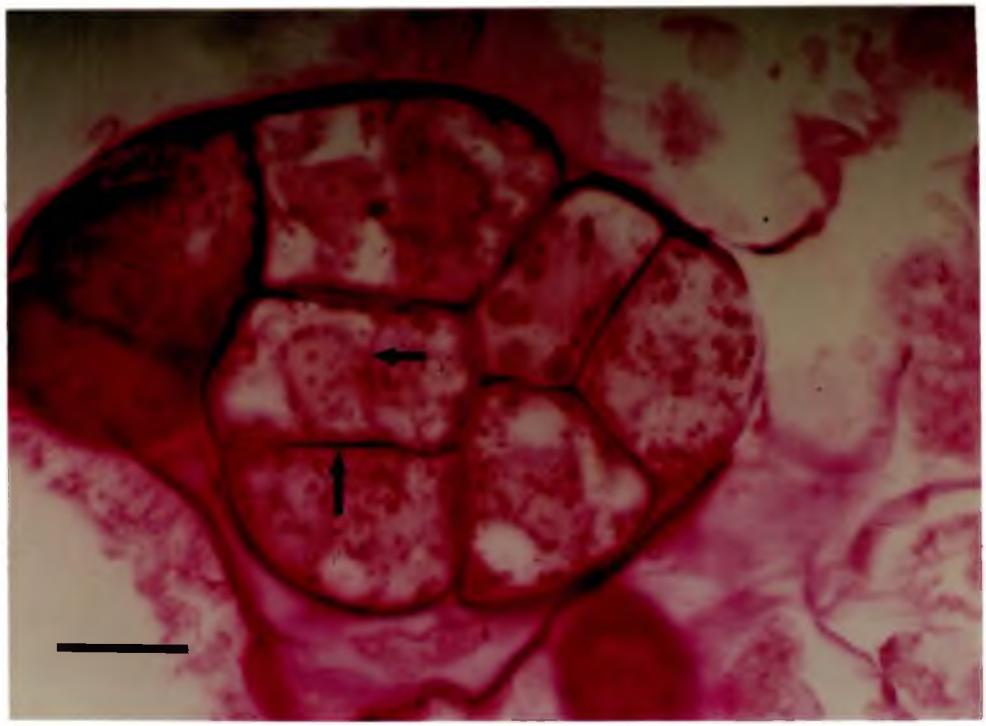


Fig. 40. Formation of promeristemoid with 7 cells cluster, stained with Feulgen-fast green. Bar represents 4 μ.

Fig. 41. Formation of meristemoid with 9 cells cluster, showing different types of division, stained with PAS. Bar represents 2 μ.

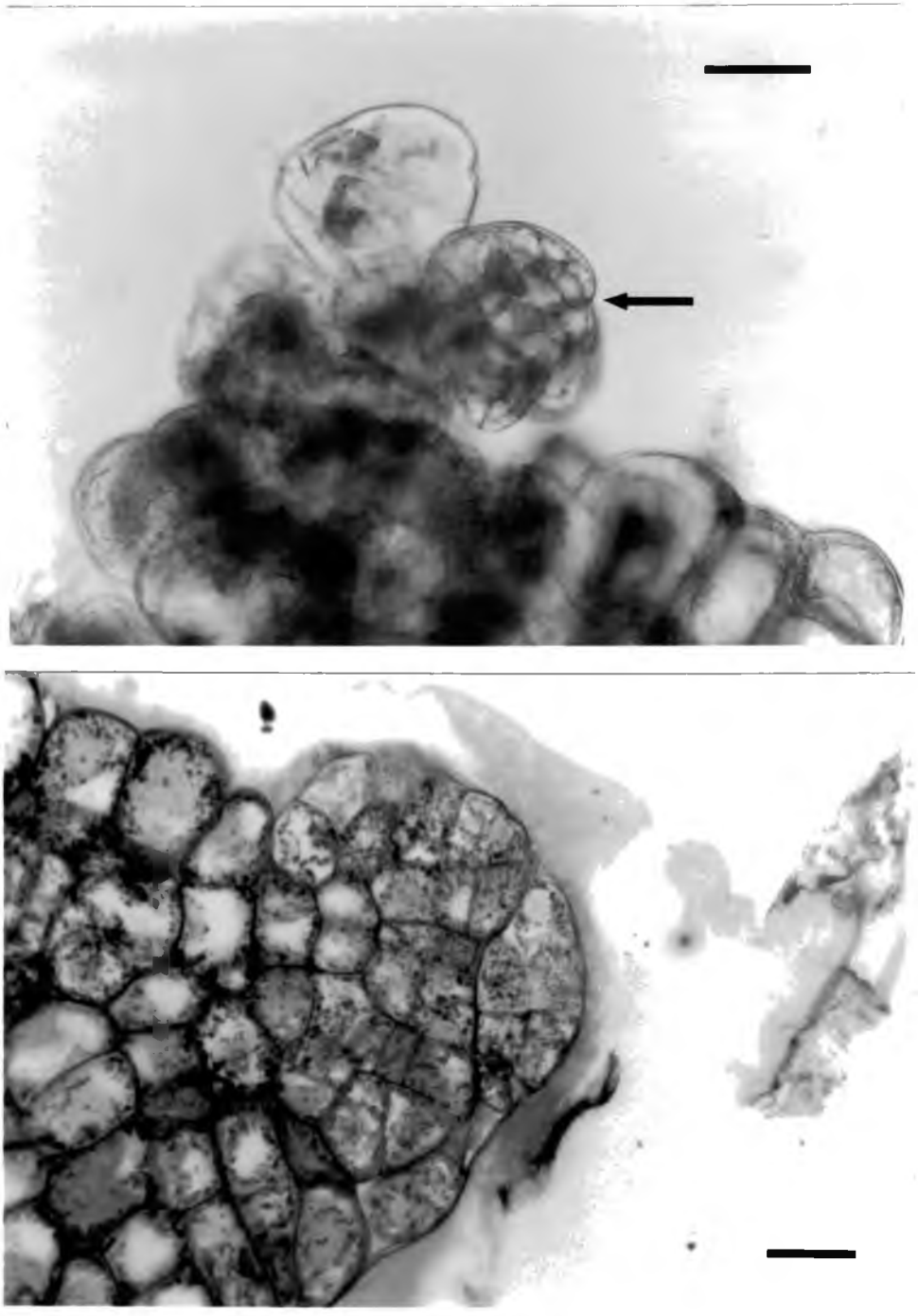
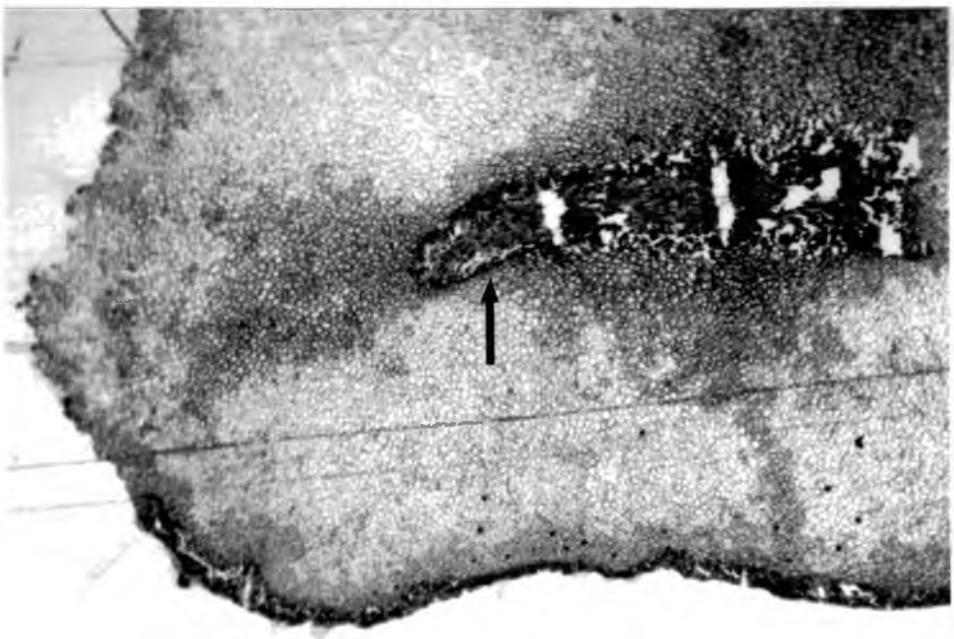


Fig. 42. Meristemoid consisting over 15 cells cluster of coconut endosperm treated with 10^{-5}M 2,4-D and BAP, stained with IKI. Bar represents 4 μ.

Fig. 43. Meristemoid consisting over 20 cells cluster of coconut endosperm treated with 10-5M 2,4-D and BAP, stained with Feulgen-fast green. Bar represents 4 μ.



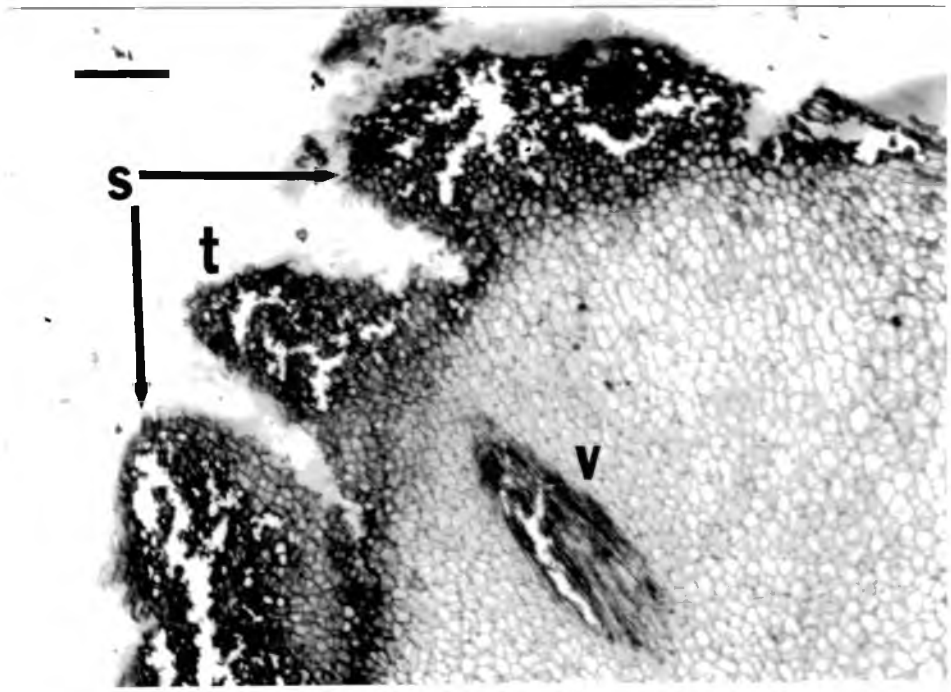


Fig. 44. Central vascular tissue of "organ-like" structure, showing parenchymatous cortex and dermal layer, stained with toluidine blue. Bar represents 100 μ.

Fig. 45. Sheating base of the cotyledon (s) and vascular tissue (v) toward stem tip (t) of "organ-like" structure, stained with toluidine blue. Bar represents 30 μ.

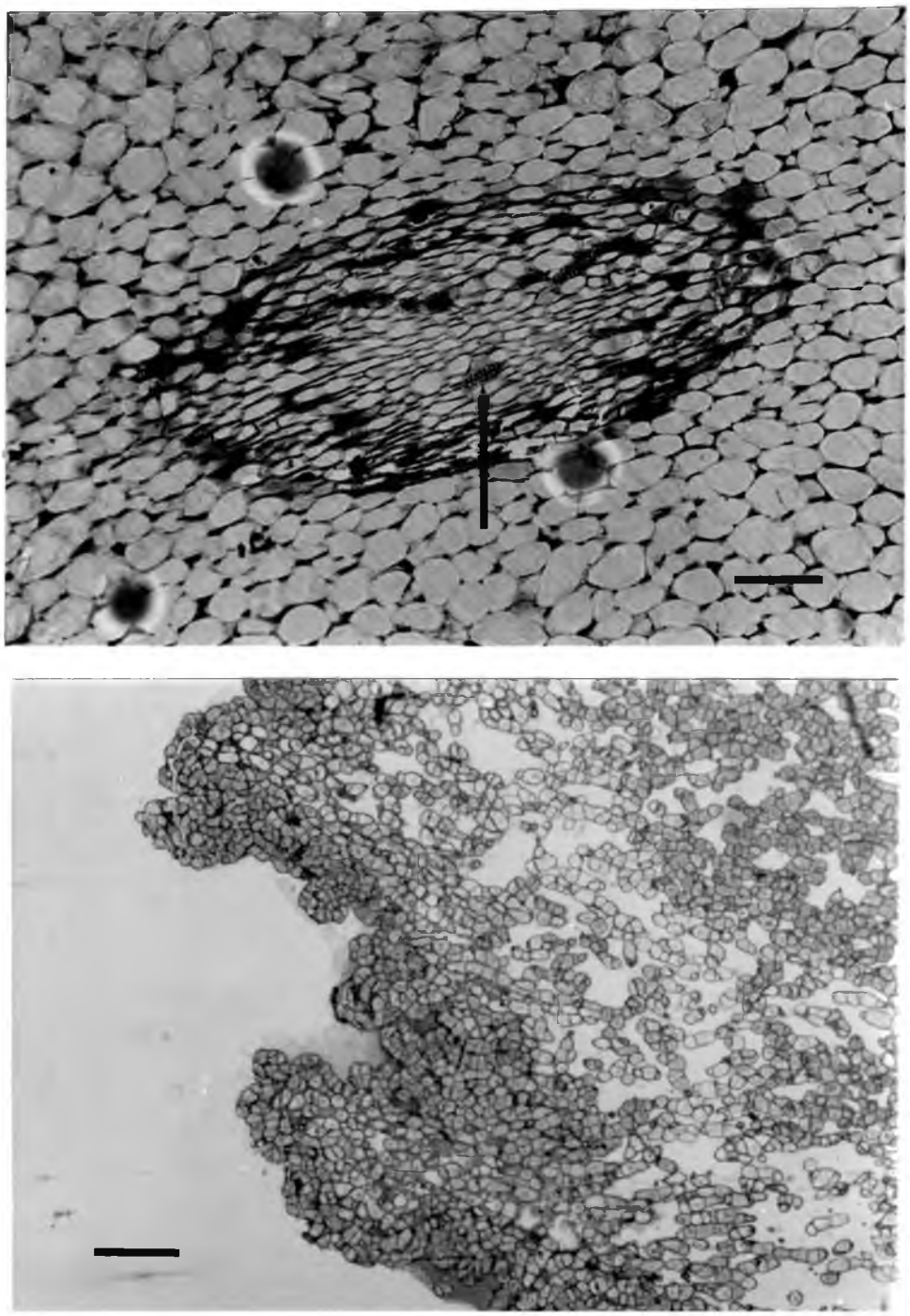


Fig. 46. Enlargement of vascular tissue showing tracheids of xylem, stained with toluidine blue. Bar represents 10 μ.

Fig. 47. Meristematic periphery of "organ-like" structure, stained with toluidine blue. Bar represents 30 μ.

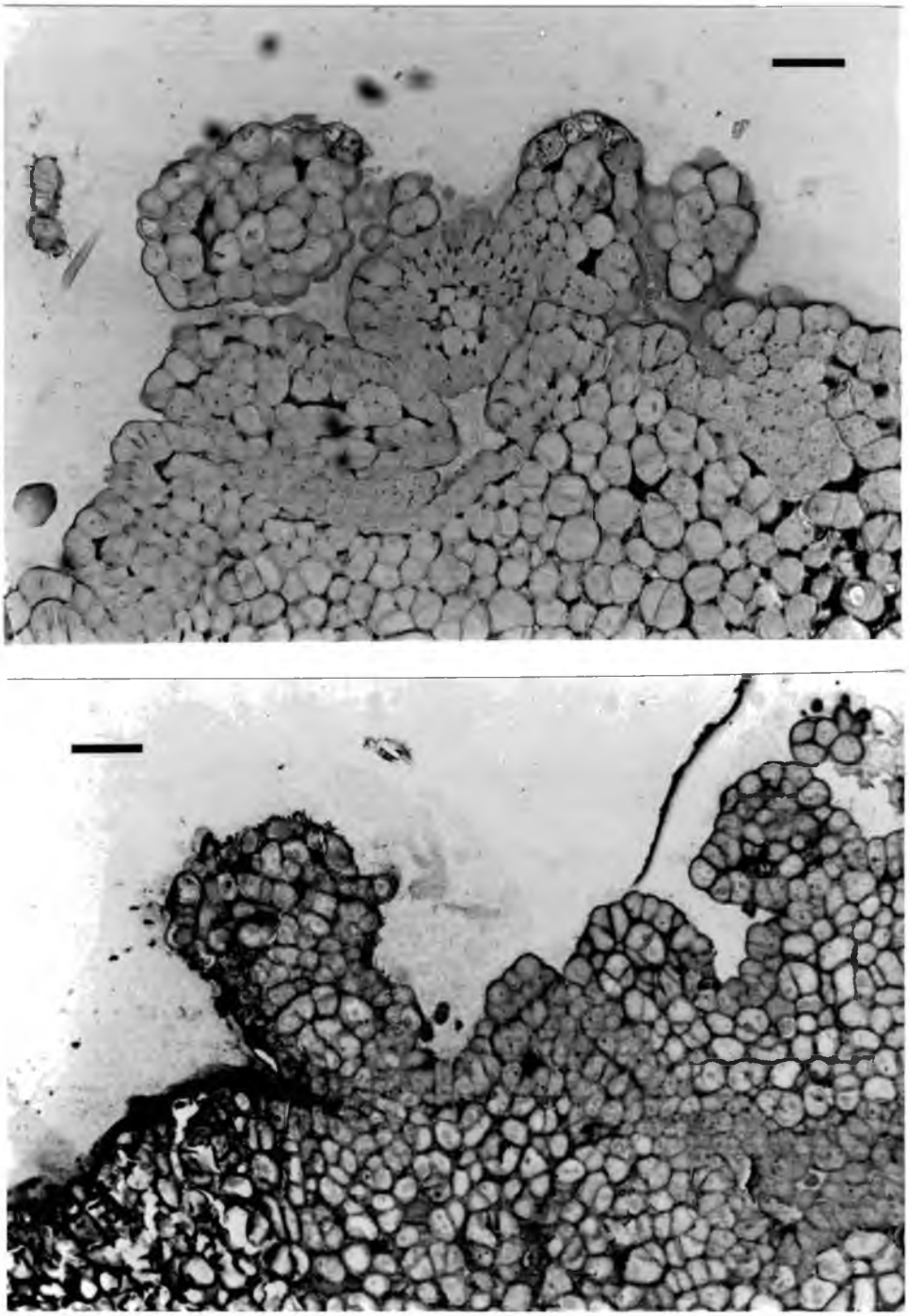


Fig. 48. Meristematic mantle of "organ-like" structure, stained with toluidine blue. Bar represents 10 μ.

Fig. 49. Protuberances of "organ-like" structure, stained with toluidine blue. Bar represents 10 μ.

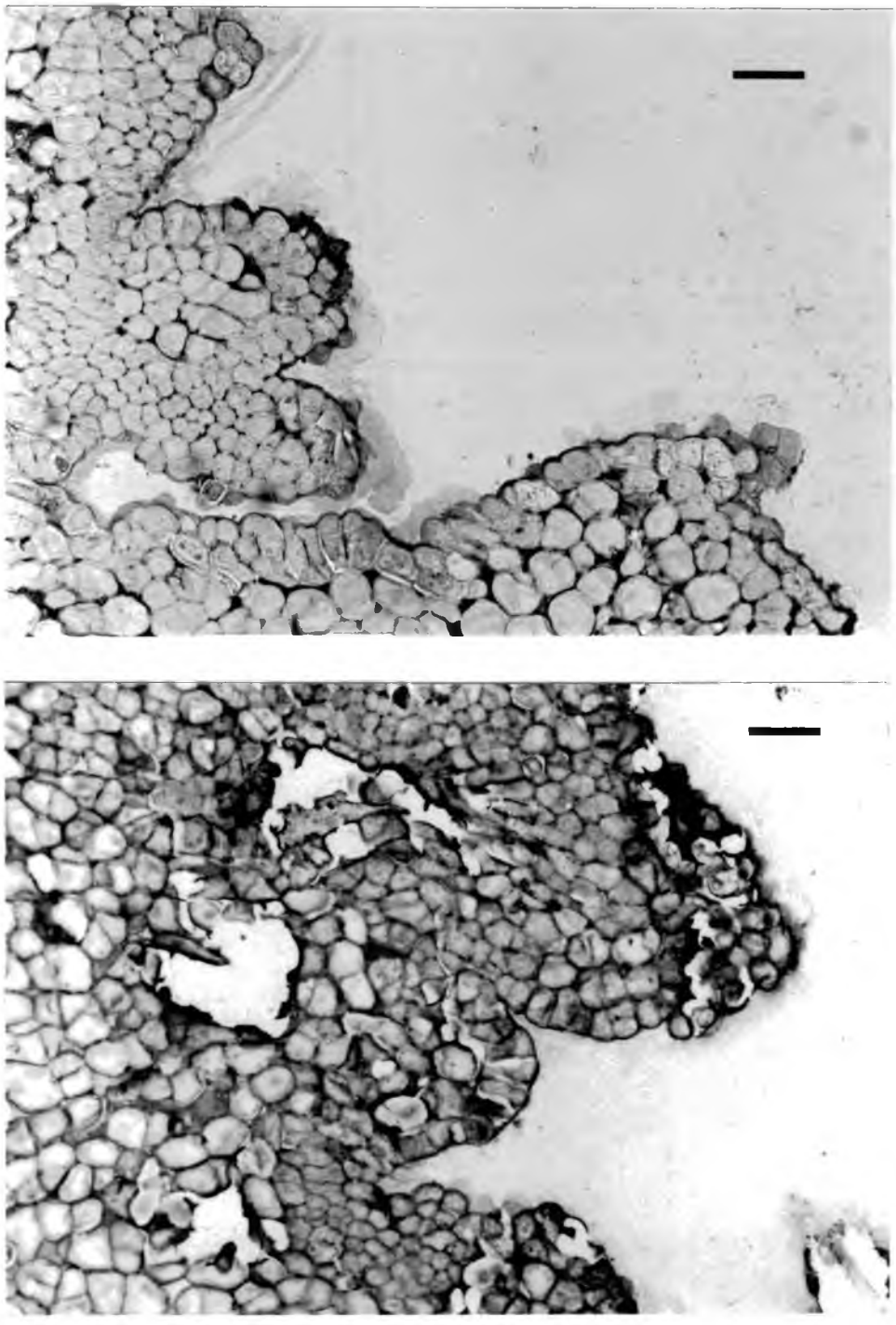


Fig. 50. Protuberances of "organ-like" structure, stained with toluidine blue. Bar represents 10 μ.

Fig. 51. Protuberances of "organ-like" structure, stained with toluidine blue. Bar represents 10 μ.

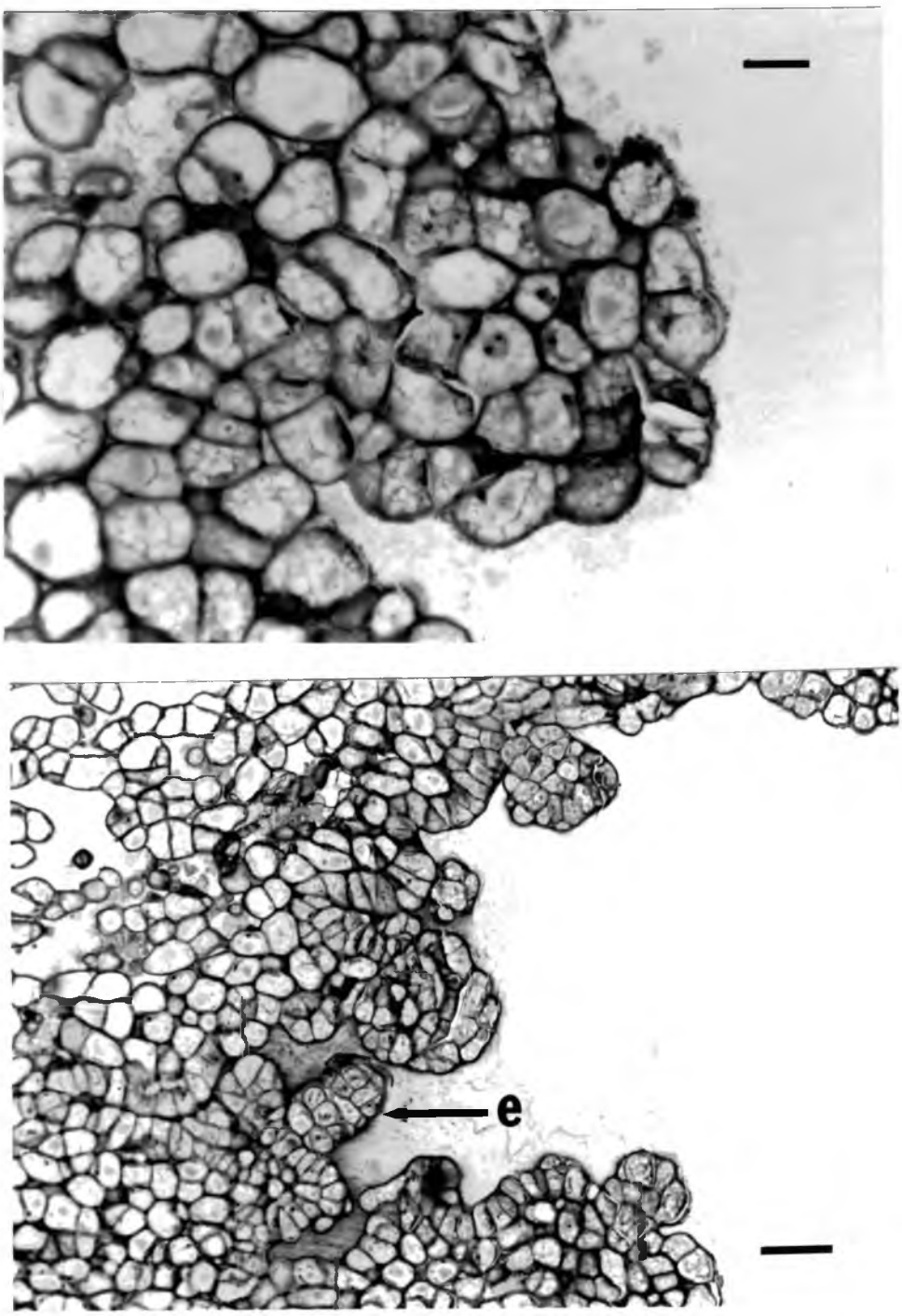


Fig. 52. Protuberance of "organ-like" structure, stained with toluidine blue. Bar represents 40 μ.

Fig. 53. Formation of embryoid (e) of "organ-like" structure, stained with toluidine blue. Bar represents 10 μ.

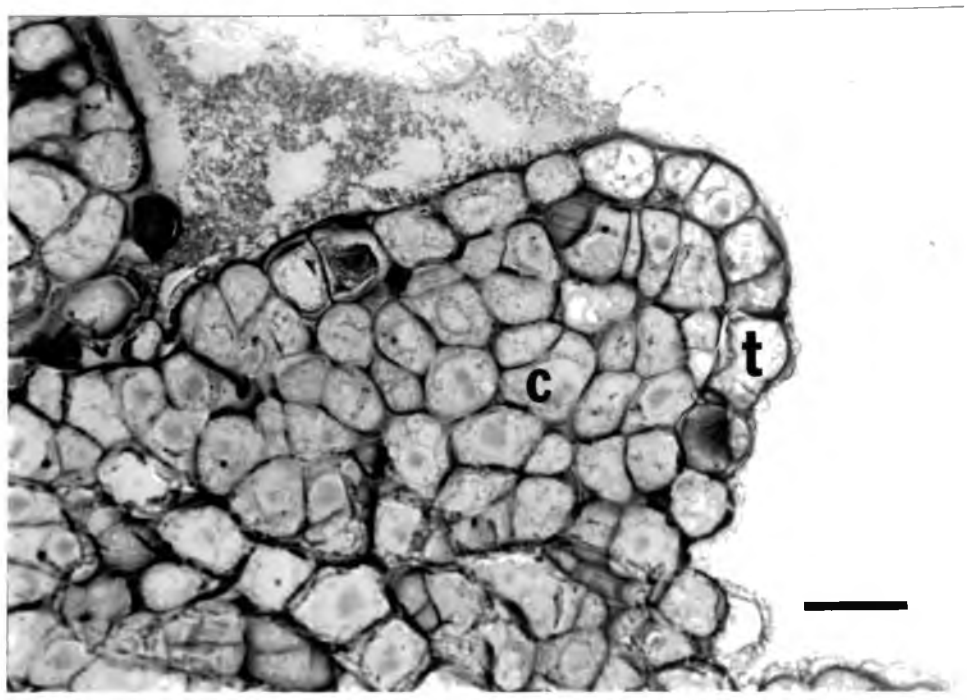


Fig. 54. Formation of one layer of tunica (t) and cell group of corpus (c) of "organ-like" structure, stained with toluidine blue. Bar represents 50 μ.

CHAPTER VI

GENERAL DISCUSSION

Callogenesis of coconut endosperm started relatively early after about 3 weeks of culture (WOC), compared to 4 weeks for Kumar et al. (1985) or over 2 months for leaf explants of coconut or oil palm (Pannetier and Buffard-Morel, 1982b; Verdeil et al., 1994).

The percentage of callogenesis was over 95% for all treatments compared to 30% in coconut endosperm for Kumar et al. (1985), 24.5% in parsley endosperm (Masuda et al., 1977), 8 - 25% for grapefruit endosperm (Gmitter et al., 1990), 1.60 - 3.74% for orange endosperm (Chen et al., 1990), 20% for leaf explants of adult coconut plants and 50% for young coconut plants (Pannetier and Buffard-Morel, 1982b), 60% - 70% for leaf explant of young coconut plants and 30% - 40% in adult coconut plants and 45% for inflorescence explants of coconut (Verdeil et al., 1989).

The high rate frequency of callogenesis obtained in by us is probably due to endogenous and exogenous factors. Endogenous factors include genotype and physiological maturity of coconut fruits (7 - 8 months postanthesis), whereas exogenous factors include disinfestant contact, culture medium, temperature and dark incubation.

Inclusion of the embryo is not essential for callus

initiation in coconut endosperm as reported in other studies of coconut (Kumar et al., 1985) as well as parsley, pear, pecan, sandalwood, walnut, Actinidia, Croton, Putranjiva and Citrus endosperm cultures (Johri and Bhojwani, 1977; Cheema and Mehra, 1982; Srivastava, 1982; Nair et al., 1986; Wang and Chang, 1978; Zhao, 1988; Mu et al., 1990).

Growth regulators were not necessary for callogenesis, possibly because coconut endosperm contained endogenous PGR, such as cytokinins and gibberellins (Radley and Dear, 1958; Zwar et al., 1963; Shaw and Srivastava, 1964; Letham, 1968). This result did not agree with Paranjothy and Rohani (1982), Reynolds (1982) and Paranjothy (1986a; 1987) who reported auxin was necessary for callogenesis in coconut culture.

Explant and callus browning occurred even with explants from young fruit, addition of activated charcoal (AC) to the medium and dark incubation. However, Dias et al. (1994) succeeded in avoiding browning in palm embryo culture of Geonoma gamiova. 2,4-D and picloram at the 10^{-4}M level or lower, as well as 10^{-5}M BAP level reduced browning. Tissue browning did not completely check tissue growth as reported in Rhododendron cultures (Ettinger and Preece, 1985).

Tissue fresh weight increased substantially with time but growth rate decreased after 26 WOC. Thus the growth of coconut callus followed a sigmoid pattern. Similar result

was reported in carrot root and sycamore cell cultures (Smith and Street, 1974; Phillips and Henshaw, 1977).

Fruit sources greatly influenced the growth response of tissues in culture. This is probably due to genotypic differences. It is well known that the genotype of explants can play a decisive role on in vitro development (Murashige, 1974). It is also possible that seasonality might have been involved because there was three months difference in the aquisition of fruits.

The growth rate of callus from the endosperm region (micropylar and antipodal tissues) did not differ significantly, even though antipodal tissues showed more browning than micropylar. This result did not agree with those of Abraham and Mathew (1963) who reported the micropylar region was more meristematic than the antipodal region. Physiological maturity of the coconut fruit might be a possible factor and they may used fruit at different maturity.

Growth rate with 2,4-D and picloram did not differ significantly. On the contrary, Fitch et al. (1986) reported 2,4-D produced faster callus growth than picloram in sugarcane hybrids and Saccharum spontaneum, while Beyl and Sharma (1983) reported picloram produced faster callus growth than 2,4-D in Gasteria and Haworthia. However, these plants are not closely related to coconut and it is not surprising that our results should differ from others.

Addition of BAP did not cause any significant difference in growth rate. These findings differ from those of Eeuwens (1978), Kuruvinashetti and Iyer (1980) and Sharma et al. (1984) who reported BAP greatly increased fresh weight of coconut and oil palm callus.

2,4-D or picloram at the 10^{-3}M level inhibited growth at the beginning but not after subculturing to 10^{-6}M. In all cases, calli were transferred to basal medium after 26 weeks of cultures. Growth rate of the control was highest at 31 WOC. Many workers (Reynolds and Murashige, 1979; Tisserat and DeMason, 1980; Blake and Eeuwens, 1982; Branton and Blake, 1983a; Gupta et al., 1984; Sharma et al., 1984; Zaid and Tisserat, 1984; Kumar et al., 1985; Karunaratne and Periyapperuma, 1989; Sugimura and Salvana, 1989; Jesty and Francis, 1992) used high concentrations of auxin to induce callogenesis in palm cultures. However, transfer to a lower concentration may be required for growth or differentiation (Murashige, 1974; Paranjothy and Rohani, 1982).

Morphogenesis occurred from antipodal tissue derived from fruit which came from the Magoon greenhouse facility of University of Hawaii at Manoa. This was initially treated with 10^{-6}M picloram and the organized structure appeared after 21 weeks. Another organized structure developed from a fruit taken from Moanalua, Oahu. It was treated with 207.04 x 10^{-6}M picloram after 17 months. This indicates that antipodal tissue has the potential to regenerate and that

picloram has a potential to induce organogenesis or embryogenesis of coconut endosperm in young and long term culture.

The "organ" was elongate and opaque. This contrasted with the surrounding yellow-brown callus. The growth of this "organ" was very slow and took about 14 months to reach a size of 9 mm diameter and 10 mm in height. During its development, it became triangular shape and then reverted to a cylindrical shape. Several lumps appeared on its surface. It superficially resembled a zygotic coconut embryo at this time (Haccius and Philip, 1979).

Histological study showed some cells of the endosperm suspensions and callus formed structures which resembled promeristemoids, meristemoids, proembryos and embryos with suspensors (Gupta and Durzan, 1987; Villalobos et al., 1985; Becwar et al., 1988; Flinn et al., 1988; Webb and Flinn, 1991).

Histological study of the "organ-like" structure showed a meristematic layer with a dermal layer, cortex-like region and central vascular tissue. There were many small protuberances which resembled embryoids and shoots with tunica corpus organization. The peripheral cells, especially in the protuberances were small, highly cytoplasmic and had prominent dark nuclei. These cells were similar to cells found in other studies Torenia fournieri and Anagallis

arvensis which become embryos, buds and shoot apices (Reinert et al., 1977).

The tunica corpus of this "organ-like" structure consisted of one layer of tunica and a large group of irregularly-arranged cells of corpus which was similar to the structure of the shoot apex of Phoenix canariensis and P. dactylifera (Ball, 1941), which are in the same family as coconut. It was likely that shoot organogenesis occurred in the endosperm calli of coconut. This is potentially important because caulogenesis has not been reported yet in coconut endosperm culture. It helps to answer the question of Blake (1990) whether shoot regeneration instead of somatic embryogenesis could occur in coconut culture.

From the preliminary experiment, a similar organ developed after 17 months of culture on the endosperm callus from tissue treated with 207.04×10^{-6}M picloram. This tissue was taken from fruit grown in Moanalua. This indicates that tissue from another genotype could produce organized growth after long time in culture.

Antiauxin, ABA, zeatin or combination of NAA, GA and BAP did not induce morphogenesis of endosperm callus.

Our work has shown that picloram has the potential for inducing differentiation of endosperm callus. Concentration and time of application of picloram are important factors for further study.

Only two occurrances of organized growth were found

from over one thousand coconut endosperm cultures. Such low frequencies are not uncommon in recalcitrant tissues. For example, callogenesis occurred in only one of several thousand anthers of coconut (Radojevic cited in Kovoor, 1981). In another study, only one embryo occurred from over 200,000 coconut anthers (Monfort, 1985). A few embryoids occurred out of a large number of oil palm (Jones, 1974) and date palm cultures (Tisserat, 1979b).

Plant regeneration has not been obtained because of the infrequency of organized growth and the long period of time that it takes to get regenerable structures. However, it is the first time organized growth of this type has been reported for coconut endosperm cultures. These results are encouraging and warrant further studies of coconut endosperm culture of coconut because in vitro regeneration has not been reported despite 40 years of work.

APPENDIX

Table 3. Analysis for percentage of callogenesis after 31 weeks of culture.

General Linear Models Procedure

Dependent Variable: CALLUS PERCENTAGE (%)

Source	DF	Sum of Squares	F Value	Pr > F
Model	18	312.94404550	0.61	0.8829
Error	125	3537.22969825		
Corrected Total	143	3850.17374375		

R-Square	C.V.	CALLUS Mean
0.081280	5.382490	98.8310417

Source	DF	Type I SS	F Value	Pr > F
BLOCK	3	45.25362986	0.53	0.6604
POS	1	0.17430625	0.01	0.9376
AUXIN	1	14.05625069	0.50	0.4823
CONC	1	25.38888951	0.90	0.3454
BA	1	32.34571436	1.14	0.2871
POS*AUXIN	1	0.17430625	0.01	0.9376
CONC*POS	1	9.91567522	0.35	0.5550
CONC*AUXIN	1	0.00000022	0.00	0.9999
CONC*BA	1	53.14056564	1.88	0.1730
POS*BA	1	20.87357511	0.74	0.3921
AUXIN*BA	1	5.33696130	0.19	0.6648
POS*AUXIN*BA	1	27.22611125	0.96	0.3285
CONC*POS*AUXIN	1	0.34282477	0.01	0.9125
CONC*POS*BA	1	1.33163653	0.05	0.8286
CONC*AUXIN*BA	1	35.14826250	1.24	0.2672
CONC*POS*AUXIN*BA	1	42.23533603	1.49	0.2241

Table 4. A summary of the percentage of callogenesis after 31 weeks of culture.

Source		Fruit 1	Fruit 2	Fruit 3	Fruit 4
Average		98.98 ± 0.71[z]a	99.44 ± 0.56a[y]	97.92 ± 1.30a	98.98 ± 0.71a
Position	A	98.89 ± 1.11a	98.89 ± 1.11a	97.69 ± 1.88a	100.00 ± 0.00a
	M	99.07 ± 0.93a	100.00 ± 0.00a	98.15 ± 1.85a	97.96 ± 1.40a
Auxin	2,4-D	98.89 ± 1.11a	100.00 ± 0.00a	96.30 ± 2.54a	98.89 ± 1.11a
	pic.	99.07 ± 0.93a	98.89 ± 1.11a	99.54 ± 0.46a	99.07 ± 0.93a
Conc.	0	100.00 ± 0.00a	100.00 ± 0.00a	97.92 ± 2.08a	100.00 ± 0.00a
	10^{-6}M	100.00 ± 0.00a	100.00 ± 0.00a	100.00 ± 0.00a	100.00 ± 0.00a
	10^{-5}M	100.00 ± 0.00a	100.00 ± 0.00a	100.00 ± 0.00a	97.92 ± 2.08a
	10^{-4}M	97.50 ± 2.50a	100.00 ± 0.00a	95.83 ± 4.17a	100.00 ± 0.00a
	10^{-3}M	97.92 ± 2.08a	97.50 ± 2.50a	95.83 ± 4.17a	97.50 ± 2.50a
BAP	0	99.00 ± 1.00a	99.00 ± 1.00a	99.58 ± 0.42a	99.00 ± 1.00a
	10^{-5}M	98.96 ± 1.04a	100.00 ± 0.00a	95.83 ± 2.85a	98.96 ± 1.04a

[z]Means ± standard error of 12 measurements.

[y]Means in the same group followed by the same letter in the columns except for the average are not significantly different at the 5% level.

Table 5. Analysis for average tissue browning on the duration of culture.

General Linear Models Procedure
Dependent Variable: BROWN RANK(0-3)

Source	DF	Sum of Squares	F Value	Pr > F
Model	4	935.92236368	296.83	0.0001
Error	4757	3749.82984128		
Corrected Total	4761	4685.75220496		

R-Square	C.V.	BROWN Mean
0.199738	47.82735	1.85636287

N P A R 1 W A Y P R O C E D U R E

Wilcoxon Scores (Rank Sums) for Variable BROWN
Classified by Variable TRANS

TRANS	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
3	896	802996.0	796992.0	9772.39018	896.200893
4	882	778535.0	784539.0	9772.39018	882.692744

Average Scores were used for Ties
Wilcoxon 2-Sample Test (Normal Approximation)
(with Continuity Correction of .5)

S= 778535 Z= -.614333 Prob > |Z| = 0.5390
T-Test approx. Significance = 0.5391

Kruskal-Wallis Test (Chi-Square Approximation)
CHISQ= 0.37747 DF= 1 Prob > CHISQ= 0.5390

Table 5. Analysis for average tissue browning on the duration of culture (continued).

TRANS	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
4	882	752247.0	747495.0	9290.34701	852.887755
5	812	683418.0	688170.0	9290.34701	841.647783

Average Scores were used for Ties
Wilcoxon 2-Sample Test (Normal Approximation)
(with Continuity Correction of .5)

S= 683418 Z= -.511445 Prob > |Z| = 0.6090

T-Test approx. Significance = 0.6091

Kruskal-Wallis Test (Chi-Square Approximation)
CHISQ= 0.26163 DF= 1 Prob > CHISQ= 0.6090

TRANS	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
2	918	703625.0	794529.0	9871.71829	766.476035
5	812	793690.0	702786.0	9871.71829	977.450739

Average Scores were used for Ties
Wilcoxon 2-Sample Test (Normal Approximation)
(with Continuity Correction of .5)

S= 793690 Z= 9.20848 Prob > |Z| = 0.0001

T-Test approx. Significance = 0.0001

Kruskal-Wallis Test (Chi-Square Approximation)
CHISQ= 84.797 DF= 1 Prob > CHISQ= 0.0001

Table 5. Analysis for average tissue browning on the duration of culture (continued).

TRANS	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	1254	1192373.0	1362471.0	13936.0271	950.85566
2	918	1167505.0	997407.0	13936.0271	1271.79194

Average Scores were used for Ties
Wilcoxon 2-Sample Test (Normal Approximation)
(with Continuity Correction of .5)

S= 1167505 Z= 12.2056 Prob > |Z| = 0.0001

T-Test approx. Significance = 0.0001

Kruskal-Wallis Test (Chi-Square Approximation)
CHISQ= 148.98 DF= 1 Prob > CHISQ= 0.0001

TRANS	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
3	896	775520.0	765632.0	9365.44788	865.535714
5	812	683966.0	693854.0	9365.44788	842.322660

Average Scores were used for Ties
Wilcoxon 2-Sample Test (Normal Approximation)
(with Continuity Correction of .5)

S= 683966 Z= -1.05574 Prob > |Z| = 0.2911

T-Test approx. Significance = 0.2912

Kruskal-Wallis Test (Chi-Square Approximation)
CHISQ= 1.1147 DF= 1 Prob > CHISQ= 0.2911

Table 5. Analysis for average tissue browning on the duration of culture (continued).

TRANS	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
2	918	715771.0	826659.0	10420.4664	779.70697
4	882	905129.0	794241.0	10420.4664	1026.22336

Average Scores were used for Ties
Wilcoxon 2-Sample Test (Normal Approximation)
(with Continuity Correction of .5)

S= 905129 Z= 10.6413 Prob > |Z| = 0.0001

T-Test approx. Significance = 0.0001

Kruskal-Wallis Test (Chi-Square Approximation)
CHISQ= 113.24 DF= 1 Prob > CHISQ= 0.0001

Table 6. A summary of browning score of tissues on the duration of culture (0 = no browning, 1 = little, 2 = medium and 3 = high).

Source		9 weeks	16 weeks	21 weeks	26 weeks	31 weeks
Average		1.18 ± 0.03[z]c	1.75 ± 0.03b[y]	2.25 ± 0.02a	2.23 ± 0.02a	2.18 ± 0.03a
Position	A	1.29 ± 0.04a	1.73 ± 0.05a	2.27 ± 0.03a	2.32 ± 0.03a	2.30 ± 0.04a
	M	1.07 ± 0.04b	1.76 ± 0.04a	2.24 ± 0.04a	2.14 ± 0.04b	2.05 ± 0.04b
Auxin	2,4-D	1.15 ± 0.04a	1.73 ± 0.05a	2.22 ± 0.03a	2.21 ± 0.04a	2.19 ± 0.04a
	pic.	1.21 ± 0.04a	1.77 ± 0.04a	2.29 ± 0.03a	2.24 ± 0.03a	2.17 ± 0.04a
Conc.	0	1.60 ± 0.06a	1.94 ± 0.06b	2.29 ± 0.05a	2.21 ± 0.04b	2.40 ± 0.07a
	10^{-6}M	0.93 ± 0.06b	1.47 ± 0.08c	1.96 ± 0.05b	2.25 ± 0.04b	2.21 ± 0.05a
	10^{-5}M	1.03 ± 0.06b	1.58 ± 0.07c	2.39 ± 0.05a	2.42 ± 0.05a	2.24 ± 0.06a
	10^{-4}M	0.95 ± 0.06b	1.64 ± 0.07c	2.27 ± 0.06a	2.09 ± 0.07b	2.17 ± 0.07a
	10^{-3}M	1.53 ± 0.07a	2.15 ± 0.07a	2.36 ± 0.05a	2.18 ± 0.06b	1.94 ± 0.07b
BAP	0		1.96 ± 0.04a	2.28 ± 0.03a	2.24 ± 0.03a	2.20 ± 0.04a
	10^{-5}M		1.43 ± 0.05b	2.22 ± 0.04a	2.21 ± 0.04a	2.15 ± 0.04a

[z]Means ± standard error of 12 measurements.

[y]Means in the same group followed by the same letter in the columns except for the average are not significantly different at the 5% level (based on a comparison of possible combinations between the average of treatments).

Table 7. Analysis for effect of transfer on growth rate.

General Linear Models Procedure

Dependent Variable: WT_GRAM

Source	DF	Sum of Squares	F Value	Pr > F
Model	4	12.28140562	62.69	0.0001
Error	675	33.05771185		
Corrected Total	679	45.33911748		

R-Square	C.V.	WT_GRAM Mean
0.270879	106.5043	0.20778647

Source	DF	Type I SS	F Value	Pr > F
TRANS	4	12.28140562	62.69	0.0001

Contrast	DF	Contrast SS	F Value	Pr > F
T1 VS T2+T3+T4+T5	1	5.35684353	109.38	0.0001
T2+T3 VS T4+T5	1	1.12654362	23.00	0.0001
T2 VS T3	1	1.04135625	21.26	0.0001
T4 VS T5	1	4.75666222	97.13	0.0001
T1 VS T2	1	1.04135625	21.26	0.0001
T3 VS T4	1	4.75666222	97.13	0.0001

Table 8. Analysis of growth rate after 9 weeks of culture.

General Linear Models Procedure

Dependent Variable: WT_GRAM FRESH WEIGHT(GRAM)

Source	DF	Sum of Squares	F Value	Pr > F
Model	10	0.04234996	5.35	0.0001
Error	68	0.05386784		
Corrected Total	78	0.09621780		

R-Square	C.V.	WT_GRAM Mean
0.440147	48.70364	0.05778949

Source	DF	Type I SS	F Value	Pr > F
BLOCK	3	0.03987013	16.78	0.0001
POS	1	0.00053882	0.68	0.4124
AUXIN	1	0.00070287	0.89	0.3496
CONC	1	0.00016710	0.21	0.6475
POS*AUXIN	1	0.00020228	0.26	0.6150
CONC*POS	1	0.00013974	0.18	0.6758
CONC*AUXIN	1	0.00048486	0.61	0.4367
CONC*POS*AUXIN	1	0.00024416	0.31	0.5806

Table 9. Analysis for effect of fruit source on growth rate after 9 weeks of culture.

General Linear Models Procedure

Dependent Variable: WT_GRAM FRESH WEIGHT(GRAM)

Source	DF	Sum of Squares	F Value	Pr > F
Model	3	0.03987013	17.69	0.0001
Error	75	0.05634767		
Corrected Total	78	0.09621780		

R-Square	C.V.	WT_GRAM Mean
0.414374	47.43058	0.05778949

Source	DF	Type I SS	F Value	Pr > F
FRT	3	0.03987013	17.69	0.0001

Contrast	DF	Contrast SS	F Value	Pr > F
F1+F2 VS F3+F4	1	0.02299825	30.61	0.0001
F1 VS F2	1	0.00336327	4.48	0.0377
F3 VS F4	1	0.01382092	18.40	0.0001
F1 VS F3	1	0.03746698	49.87	0.0001
F1 VS F4	1	0.00600911	8.00	0.0060
F2 VS F3	1	0.01886295	25.11	0.0001
F2 VS F4	1	0.00039125	0.52	0.4728

Table 10. Analysis for effect of 2,4-D and picloram concentrations (C) on growth rate after 9 weeks of culture.

General Linear Models Procedure

Dependent Variable: WT_GRAM FRESH WEIGHT(GRAM)

Source	DF	Sum of Squares	F Value	Pr > F
Model	4	0.02758916	7.44	0.0001
Error	74	0.06862863		
Corrected Total	78	0.09621780		

R-Square	C.V.	WT_GRAM Mean
0.286737	52.69725	0.05778949

Source	DF	Type I SS	F Value	Pr > F
CONC	4	0.02758916	7.44	0.0001

Contrast	DF	Contrast SS	F Value	Pr > F
C0 VS C3+C4+C5+C6	1	0.00334743	3.61	0.0613
C3+C4 VS C5+C6	1	0.00750451	8.09	0.0057
C3 VS C4	1	0.01573538	16.97	0.0001
C5 VS C6	1	0.00084362	0.91	0.3433
C0 VS C6	1	0.00000003	0.00	0.9955
C0 VS C5	1	0.00085345	0.92	0.3405
C0 VS C4	1	0.00019538	0.21	0.6476
C0 VS C3	1	0.01943751	20.96	0.0001
C3 VS C5	1	0.01165051	12.56	0.0007
C3 VS C6	1	0.01938973	20.91	0.0001
C4 VS C5	1	0.00023912	0.26	0.6131
C4 VS C6	1	0.00019061	0.21	0.6516

Table 11. Analysis of growth rate after 16 weeks of culture.

General Linear Models Procedure

Dependent Variable: WT_GRAM FRESH WEIGHT(GRAM)

Source	DF	Sum of Squares	F Value	Pr > F
Model	18	0.93309322	12.77	0.0001
Error	125	0.50754324		
Corrected Total	143	1.44063646		

R-Square	C.V.	WT_GRAM Mean
0.647695	44.23011	0.14406667

Source	DF	Type I SS	F Value	Pr > F
BLOCK	3	0.86936648	71.37	0.0001
POS	1	0.00332160	0.82	0.3675
AUXIN	1	0.00250333	0.62	0.4338
CONC	1	0.02427858	5.98	0.0159
BA	1	0.00152249	0.37	0.5414
POS*AUXIN	1	0.00651787	1.61	0.2075
CONC*POS	1	0.00146379	0.36	0.5493
CONC*AUXIN	1	0.00122761	0.30	0.5834
CONC*BA	1	0.00000000	0.00	0.9996
POS*BA	1	0.00004600	0.01	0.9154
AUXIN*BA	1	0.00004693	0.01	0.9146
POS*AUXIN*BA	1	0.00446233	1.10	0.2965
CONC*POS*AUXIN	1	0.00605424	1.49	0.2243
CONC*POS*BA	1	0.00030964	0.08	0.7829
CONC*AUXIN*BA	1	0.00060461	0.15	0.7002
CONC*POS*AUXIN*BA	1	0.01136771	2.80	0.0968

Table 12. Analysis for effect of fruit source on growth rate after 16 weeks of culture.

General Linear Models Procedure

Dependent Variable: WT_GRAM FRESH WEIGHT(GRAM)

Source	DF	Sum of Squares	F Value	Pr > F
Model	3	0.86936648	71.02	0.0001
Error	140	0.57126998		
Corrected Total	143	1.44063646		

R-Square	C.V.	WT_GRAM Mean
0.603460	44.33974	0.14406667

Source	DF	Type I SS	F Value	Pr > F
FRT	3	0.86936648	71.02	0.0001

Source	DF	Type III SS	F Value	Pr > F
FRT	3	0.86936648	71.02	0.0001

Contrast	DF	Contrast SS	F Value	Pr > F
F1+F2 VS F3+F4	1	0.71008711	174.02	0.0001
F1 VS F2	1	0.00073856	0.18	0.6712
F3 VS F4	1	0.15854081	38.85	0.0001
F1 VS F3	1	0.65371989	160.21	0.0001
F1 VS F4	1	0.16839339	41.27	0.0001
F2 VS F3	1	0.61051250	149.62	0.0001
F2 VS F4	1	0.14682780	35.98	0.0001

Table 13. Analysis for effect of 2,4-D and picloram concentrations (C) on growth rate after 16 weeks of culture.

General Linear Models Procedure

Dependent Variable: WT_GRAM FRESH WEIGHT(GRAM)

Source	DF	Sum of Squares	F Value	Pr > F
Model	4	0.05778758	1.45	0.2201
Error	139	1.38284888		
Corrected Total	143	1.44063646		

R-Square	C.V.	WT_GRAM Mean
0.040113	69.23353	0.14406667

Source	DF	Type I SS	F Value	Pr > F
CONC	4	0.05778758	1.45	0.2201

Contrast	DF	Contrast SS	F Value	Pr > F
C0 VS C3+C4+C5+C6	1	0.00266450	0.27	0.6056
C3+C4 VS C5+C6	1	0.03021497	3.04	0.0836
C3 VS C4	1	0.02338223	2.35	0.1275
C5 VS C6	1	0.00152588	0.15	0.6959
C0 VS C6	1	0.00623070	0.63	0.4301
C0 VS C5	1	0.01228311	1.23	0.2684
C0 VS C4	1	0.00324338	0.33	0.5689
C0 VS C3	1	0.00461067	0.46	0.4971
C3 VS C5	1	0.04791721	4.82	0.0298
C3 VS C6	1	0.03234153	3.25	0.0736
C4 VS C5	1	0.00435435	0.44	0.5093
C4 VS C6	1	0.00072496	0.07	0.7876

Table 14. Analysis of growth rate after 21 weeks of culture.

General Linear Models Procedure

Dependent Variable: WT_GRAM FRESH WEIGHT(GRAM)

Source	DF	Sum of Squares	F Value	Pr > F
Model	18	6.30285409	61.18	0.0001
Error	125	0.71541530		
Corrected Total	143	7.01826939		

R-Square	C.V.	WT_GRAM Mean
0.898064	28.21770	0.26810347

Source	DF	Type I SS	F Value	Pr > F
BLOCK	3	6.23490894	363.13	0.0001
POS	1	0.00585863	1.02	0.3136
AUXIN	1	0.00116793	0.20	0.6522
CONC	1	0.00239390	0.42	0.5190
BA	1	0.00783956	1.37	0.2441
POS*AUXIN	1	0.00200182	0.35	0.5553
CONC*POS	1	0.00036939	0.06	0.7999
CONC*AUXIN	1	0.00067424	0.12	0.7320
CONC*BA	1	0.00047060	0.08	0.7748
POS*BA	1	0.00407002	0.71	0.4007
AUXIN*BA	1	0.00017810	0.03	0.8603
POS*AUXIN*BA	1	0.00000297	0.00	0.9819
CONC*POS*AUXIN	1	0.02755768	4.81	0.0301
CONC*POS*BA	1	0.00357565	0.62	0.4308
CONC*AUXIN*BA	1	0.00103925	0.18	0.6708
CONC*POS*AUXIN*BA	1	0.01074540	1.88	0.1731

Table 15. Analysis for effect of fruit source on growth rate after 21 weeks of culture.

General Linear Models Procedure

Dependent Variable: WT_GRAM FRESH WEIGHT(GRAM)

Source	DF	Sum of Squares	F Value	Pr > F
Model	3	6.23490894	371.43	0.0001
Error	140	0.78336044		
Corrected Total	143	7.01826939		

R-Square	C.V.	WT_GRAM Mean
0.888383	27.90065	0.26810347

Source	DF	Type I SS	F Value	Pr > F
FRT	3	6.23490894	371.43	0.0001

Source	DF	Type III SS	F Value	Pr > F
FRT	3	6.23490894	371.43	0.0001

Contrast	DF	Contrast SS	F Value	Pr > F
F1+F2 VS F3+F4	1	6.05213301	1081.62	0.0001
F1 VS F2	1	0.14857609	26.55	0.0001
F3 VS F4	1	0.03419984	6.11	0.0146
F1 VS F3	1	4.09962523	732.67	0.0001
F1 VS F4	1	3.38494181	604.95	0.0001
F2 VS F3	1	2.68729608	480.27	0.0001
F2 VS F4	1	2.11517884	378.02	0.0001

Table 16. Analysis for effect of 2,4-D and picloram concentrations (C) on growth rate after 21 weeks of culture.

General Linear Models Procedure

Dependent Variable: WT_GRAM

Source	DF	Sum of Squares	F Value	Pr > F
Model	4	0.03919786	0.20	0.9406
Error	139	6.97907153		
Corrected Total	143	7.01826939		

R-Square	C.V.	WT_GRAM Mean
0.005585	83.57742	0.26810347

Source	DF	Type I SS	F Value	Pr > F
CONC	4	0.03919786	0.20	0.9406

Contrast	DF	Contrast SS	F Value	Pr > F
C0 VS C3+C4+C5+C6	1	0.00095357	0.02	0.8906
C3+C4 VS C5+C6	1	0.00007488	0.00	0.9693
C3 VS C4	1	0.03664353	0.73	0.3944
C5 VS C6	1	0.00152588	0.03	0.8619
C0 VS C6	1	0.00006885	0.00	0.9705
C0 VS C5	1	0.00161540	0.03	0.8579
C0 VS C4	1	0.01153255	0.23	0.6325
C0 VS C3	1	0.00239201	0.05	0.8275
C3 VS C5	1	0.01190827	0.24	0.6270
C3 VS C6	1	0.00490875	0.10	0.7550
C4 VS C5	1	0.00677329	0.13	0.7140
C4 VS C6	1	0.01472886	0.29	0.5889

Table 17. Analysis of growth rate after 26 weeks of culture.

General Linear Models Procedure

Dependent Variable: WT_GRAM FRESH WEIGHT(GRAM)

Source	DF	Sum of Squares	F Value	Pr > F
Model	18	19.99872751	65.08	0.0001
Error	125	2.13391013		
Corrected Total	143	22.13263764		

R-Square	C.V.	WT_GRAM Mean
0.903585	30.36890	0.43023333

Source	DF	Type I SS	F Value	Pr > F
BLOCK	3	19.79017927	386.42	0.0001
POS	1	0.01164241	0.68	0.4105
AUXIN	1	0.00704201	0.41	0.5219
CONC	1	0.00000587	0.00	0.9852
BA	1	0.03821031	2.24	0.1372
POS*AUXIN	1	0.01402251	0.82	0.3665
CONC*POS	1	0.01250917	0.73	0.3936
CONC*AUXIN	1	0.00000090	0.00	0.9942
CONC*BA	1	0.00781517	0.46	0.4999
POS*BA	1	0.00280599	0.16	0.6859
AUXIN*BA	1	0.00062594	0.04	0.8485
POS*AUXIN*BA	1	0.08183149	4.79	0.0304
CONC*POS*AUXIN	1	0.00828902	0.49	0.4872
CONC*POS*BA	1	0.01602825	0.94	0.3344
CONC*AUXIN*BA	1	0.00397960	0.23	0.6301
CONC*POS*AUXIN*BA	1	0.00373961	0.22	0.6406

Table 18. Analysis for effect of fruit source on growth rate after 26 weeks of culture.

General Linear Models Procedure

Dependent Variable: WT_GRAM FRESH WEIGHT(GRAM)

Source	DF	Sum of Squares	F Value	Pr > F
Model	3	19.79017927	394.26	0.0001
Error	140	2.34245837		
Corrected Total	143	22.13263764		

R-Square	C.V.	WT_GRAM Mean
0.894163	30.06546	0.43023333

Source	DF	Type I SS	F Value	Pr > F
FRT	3	19.79017927	394.26	0.0001

Source	DF	Type III SS	F Value	Pr > F
FRT	3	19.79017927	394.26	0.0001

Contrast	DF	Contrast SS	F Value	Pr > F
F1+F2 VS F3+F4	1	19.76350755	1181.19	0.0001
F1 VS F2	1	0.02439472	1.46	0.2293
F3 VS F4	1	0.00227700	0.14	0.7128
F1 VS F3	1	10.53313202	629.53	0.0001
F1 VS F4	1	10.22567402	611.15	0.0001
F2 VS F3	1	9.54371642	570.39	0.0001
F2 VS F4	1	9.25116436	552.91	0.0001

Table 19. Analysis for effect of 2,4-D and picloram concentrations (C) on growth rate after 26 weeks of culture.

General Linear Models Procedure

Dependent Variable: WT_GRAM

Source	DF	Sum of Squares	F Value	Pr > F
Model	4	0.04466759	0.07	0.9909
Error	139	22.08797005		
Corrected Total	143	22.13263764		

R-Square	C.V.	WT_GRAM Mean
0.002018	92.65449	0.43023333

Source	DF	Type I SS	F Value	Pr > F
CONC	4	0.04466759	0.07	0.9909

Contrast	DF	Contrast SS	F Value	Pr > F
C0 VS C3+C4+C5+C6	1	0.00054162	0.00	0.9535
C3+C4 VS C5+C6	1	0.00430940	0.03	0.8694
C3 VS C4	1	0.00981833	0.06	0.8041
C5 VS C6	1	0.02999824	0.19	0.6646
C0 VS C6	1	0.00517147	0.03	0.8571
C0 VS C5	1	0.00483084	0.03	0.8618
C0 VS C4	1	0.00000181	0.00	0.9973
C0 VS C3	1	0.00632938	0.04	0.8421
C3 VS C5	1	0.03332907	0.21	0.6477
C3 VS C6	1	0.00008766	0.00	0.9813
C4 VS C5	1	0.00696808	0.04	0.8344
C4 VS C6	1	0.00805058	0.05	0.8222

Table 20. Analysis of growth rate after 31 weeks of culture.

General Linear Models Procedure

Dependent Variable: WT_GRAM FRESH WEIGHT(GRAM)

Source	DF	Sum of Squares	F Value	Pr > F
Model	18	3.05824230	6.28	0.0001
Error	125	3.38033312		
Corrected Total	143	6.43857542		

R-Square	C.V.	WT_GRAM Mean
0.474987	90.10049	0.18251458

Source	DF	Type I SS	F Value	Pr > F
BLOCK	3	1.49355537	18.41	0.0001
POS	1	0.00002678	0.00	0.9749
AUXIN	1	0.00330721	0.12	0.7271
CONC	1	0.95558243	35.34	0.0001
BA	1	0.06700208	2.48	0.1180
POS*AUXIN	1	0.01309690	0.48	0.4878
CONC*POS	1	0.00500493	0.19	0.6678
CONC*AUXIN	1	0.29351937	10.85	0.0013
CONC*BA	1	0.09350959	3.46	0.0653
POS*BA	1	0.01215968	0.45	0.5037
AUXIN*BA	1	0.00220087	0.08	0.7759
POS*AUXIN*BA	1	0.00027541	0.01	0.9198
CONC*POS*AUXIN	1	0.02104024	0.78	0.3794
CONC*POS*BA	1	0.00043161	0.02	0.8997
CONC*AUXIN*BA	1	0.09627214	3.56	0.0615
CONC*POS*AUXIN*BA	1	0.00125771	0.05	0.8296

Table 21. Analysis for effect of fruit source on growth rate after 31 weeks of culture.

General Linear Models Procedure

Dependent Variable: WT_GRAM FRESH WEIGHT(GRAM)

Source	DF	Sum of Squares	F Value	Pr > F
Model	3	1.49355537	14.09	0.0001
Error	140	4.94502004		
Corrected Total	143	6.43857542		

R-Square	C.V.	WT_GRAM Mean
0.231970	102.9728	0.18251458

Source	DF	Type I SS	F Value	Pr > F
FRT	3	1.49355537	14.09	0.0001

Source	DF	Type III SS	F Value	Pr > F
FRT	3	1.49355537	14.09	0.0001

Contrast	DF	Contrast SS	F Value	Pr > F
F1+F2 VS F3+F4	1	1.45827763	41.29	0.0001
F1 VS F2	1	0.01520768	0.43	0.5128
F3 VS F4	1	0.02007006	0.57	0.4522
F1 VS F3	1	0.52042102	14.73	0.0002
F1 VS F4	1	0.74489149	21.09	0.0001
F2 VS F3	1	0.71355449	20.20	0.0001
F2 VS F4	1	0.97296600	27.55	0.0001

Table 22. Analysis for effect of 2,4-D and picloram concentrations (C) on growth rate after 31 weeks of culture.

General Linear Models Procedure

Dependent Variable: WT_GRAM FRESH WEIGHT(GRAM)

Source	DF	Sum of Squares	F Value	Pr > F
Model	4	1.61868442	11.67	0.0001
Error	139	4.81989100		
Corrected Total	143	6.43857542		

R-Square	C.V.	WT_GRAM Mean
0.251404	102.0267	0.18251458

Source	DF	Type I SS	F Value	Pr > F
CONC	4	1.61868442	11.67	0.0001

Contrast	DF	Contrast SS	F Value	Pr > F
C0 VS C3+C4+C5+C6	1	1.51861057	43.79	0.0001
C3+C4 VS C5+C6	1	0.01966392	0.57	0.4527
C3 VS C4	1	0.07618980	2.20	0.1405
C5 VS C6	1	0.00422013	0.12	0.7277
C0 VS C6	1	1.10935700	31.99	0.0001
C0 VS C5	1	1.00043708	28.85	0.0001
C0 VS C4	1	1.48934399	42.95	0.0001
C0 VS C3	1	0.99005157	28.55	0.0001
C3 VS C5	1	0.00004064	0.00	0.9727
C3 VS C6	1	0.00508904	0.15	0.7022
C4 VS C5	1	0.07271112	2.10	0.1498
C4 VS C6	1	0.04189697	1.21	0.2736

Table 23. A summary of growth rate (g per week) of tissues on the duration of culture.

Source		9 weeks	16 weeks	21 weeks	26 weeks	31 weeks
Average		0.030±0.039[z]e	0.145±0.102d[y]	0.268±0.221b	0.430±0.394a	0.165±0.169c
Fruit	1	0.084±0.007a	0.217±0.009a	0.518±0.012a	0.819±0.028a	0.269±0.045a
	2	0.066±0.008b	0.211±0.017a	0.428±0.021b	0.782±0.032a	0.298±0.042a
	3	0.022±0.003c	0.027±0.004c	0.041±0.004d	0.054±0.004b	0.099±0.008b
	4	0.060±0.005b	0.121±0.009b	0.085±0.005c	0.065±0.005b	0.065±0.006b
Position	A	0.060±0.006a	0.149±0.012a	0.274±0.027a	0.439±0.047a	0.182±0.024a
	M	0.055±0.005a	0.139±0.012a	0.262±0.025a	0.421±0.046a	0.183±0.026a
Auxin	2,4-D	0.061±0.006a	0.148±0.012a	0.265±0.026a	0.437±0.046a	0.187±0.022a
	pic.	0.054±0.005a	0.140±0.012a	0.271±0.027a	0.423±0.046a	0.178±0.028a
Conc.	0	0.071±0.008a	0.132±0.018ab	0.261±0.056a	0.436±0.098a	0.473±0.111a
	10^{-6}M	0.071±0.008a	0.156±0.016ab	0.263±0.038a	0.414±0.065a	0.150±0.028b
	10^{-5}M	0.060±0.007a	0.166±0.015a	0.273±0.037a	0.457±0.074a	0.167±0.023b
	10^{-4}M	0.066±0.010a	0.149±0.016ab	0.294±0.043a	0.436±0.074a	0.099±0.011b
	10^{-3}M	0.021±0.003b	0.111±0.024 b	0.246±0.040a	0.411±0.070a	0.168±0.021b
BAP	0		0.138±0.010a	0.261±0.024a	0.444±0.046a	0.200±0.030a
	10^{-5}M		0.151±0.014 a	0.277±0.028 a	0.413±0.046a	0.136±0.013a

[z]Means ± standar error of 12 measurements.

[y]Means in the same group followed by the same letter in the columns except for the average are not significantly different at the 5% level.

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